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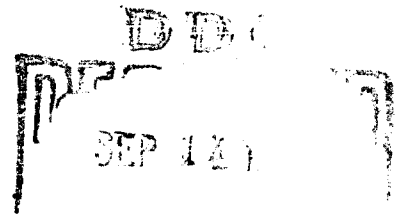
TECHNICAL MANUSCRIPT 233

**LETHAL PROCESSES
IN DEHYDRATION OF MICROORGANISMS**

L.B. Bateman

AUGUST 1965

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**UNITED STATES ARMY
BIOLOGICAL LABORATORIES
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U.S. ARMY BIOLOGICAL LABORATORIES
Fort Detrick, Frederick, Maryland

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LETHAL PROCESSES IN DEHYDRATION OF MICROORGANISMS

J.B. Bateman

Physical Sciences Division
DIRECTORATE OF BIOLOGICAL RESEARCH

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ABSTRACT

Approaches to the study of lethal effects of dehydration and relevant experimental results obtained by the author and co-workers since 1958 are summarized informally. The paper includes (i) a working hypothesis of interacting lethal processes from which self-limiting long-term decay curves can be calculated. (ii) Preliminary experimental data illustrating the types of information needed for application of the hypothesis. (iii) An account of the preparation of "simulated aerosols" by deposition of cells upon membrane filters. (iv) Approaches to the identification of lethal interactions, including the correlation of lethal effects with the colligative properties of simple additives. (v) Illustrative data for Serratia marcescens in the presence of NaCl, LiBr, and urea. (vi) Data on short-term decay of washed Serratia marcescens at 13 ambient relative humidities. (vii) Preliminary data on the effects of oxygen in accelerating humidity-dependent decay and on possible effects of slow residual water transfer.

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DIGEST

This paper contains an informal statement of the ideas that have guided our laboratory work on the survival of dehydrated microorganisms, and a preliminary summary of experimental results obtained since June 1958.

The need for working hypotheses concerning lethal processes is stressed, and the idea of interacting intracellular changes resulting in self-limiting decay curves is developed briefly. It is maintained that hypotheses of this kind may serve as a basis for fruitful experimentation directed toward the identification of lethal mechanisms.

It is argued that in research relating to the stability of microbial aerosols there is need for the study of isolated contributory processes, and the effects of lowered relative humidity are shown to be amenable to examination by very simple, inexpensive techniques involving deposition of cells upon filter paper. The decay kinetics of such simulated aerosols are attributable, directly or indirectly, to water loss, with relatively small contributions from the other stresses that accompany ordinary aerosolization.

Possible approaches to the identification of lethal interactions include both physicochemical and biological modification of cells prior to, or subsequent to, the examination of the decay kinetics at reduced relative humidity, as well as the examination of organisms that are unusually sensitive or unusually resistant to related stresses such as extremes of temperature, dryness, or salt concentration. Particular importance is attached to the use, in studies of relative humidity, of physicochemically well defined additives. Since such substances usually have limited solubility, there should be a cut-off point in their biological effectiveness as the relative humidity is reduced, and failure to observe such a cut-off may provide a clue to the properties of the additive in its biologically active (combined) form. Finally, the importance of long-term decay measurements, which would not be feasible in conventional aerosol studies, is emphasized in any appraisal of the effects of additives.

The decay curves over limited periods (up to 72 hours) for washed Serratia marcescens 8 UK deposited on membrane filters are reported for 13 values of the ambient relative humidity in air. There is a general increase in initial decay rate with decreasing humidity; this rapid initial decay rate is succeeded by slower decay with much less marked humidity dependence.

The predicted cut-off phenomenon has been examined for cells in presence of sodium chloride, lithium bromide, and urea. In each case a cut-off was observed but it was gradual, and it did not occur at the predicted relative humidity. All three substances appear to have a slight stabilizing effect at zero humidity. It is predicted that, in

general, the effects of sensitizing or stabilizing additives will be humidity-dependent and not necessarily related in any simple manner to their behavior in aqueous solution.

Several long-term decay curves, for exposures up to 26 days, are presented to illustrate the procedure adopted for checking conformity with theoretical equations. Poor reproducibility continues to hamper this operation.

Experiments are described in which slow residual water loss from dried cells was prevented by covering the cells with mercury. The effect, however, appeared to be due largely to the concomitant exclusion of air. During submersion in mercury or exposure to vacuum the cells became sensitized, so that accelerated decay occurred upon subsequent access to air. This is explained in terms of accumulation of an oxidizable precursor of a lethal substance.

Several physicochemical properties of cells relevant to the dehydration problem are discussed briefly. It is thought that, in particular, dielectric dispersion measurements and determination of the colligative properties of normal and added solutes in the presence of cell substance should be particularly powerful tools in examining the properties of the intracellular continuum of normal and dehydrated cells.

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I. INTRODUCTION: AN APPROACH TO THE STUDY OF AIRBORNE INFECTION

In the long chain of processes involved in airborne infection, a few of which are listed in Table 1, many can be considered to involve stress upon the infectious agent, the effects of which can only be manifested in terms of biological end results if they are able to bring about some intracellular event. We can hardly expect to be able to understand or control the course of airborne infection unless we have some understanding of these events, and our success in reaching such understanding is not likely to be at all impressive unless we can isolate the individual stresses, take them apart, so to speak, and study them in depth, no matter how far this may take us from the employment of professional sneezers, or from the entrapment of aerosol particles or the stationing of guinea pigs at the entrances to subway stations. In this interim report, I shall assume that you sympathize with this wish to isolate the components of a very complex process, and shall try to point out some of the difficult problems that remain even when one has gone about as far as possible in isolating and simplifying the system for investigation.

It has long been realized that a loss and uptake of water even by primitive organisms may have quite profound biological effects, and in electing to study these processes as they are triggered by the transfer of water we were mindful of the fact that changes of water content probably occur at every stage of airborne infection listed in Table 1, from the ejection of the organism into a comparatively hostile atmosphere to its arrival at a comfortable destination within the host, so that water may be of paramount significance throughout.

TABLE 1. PROCESSES IN AIRBORNE INFECTION

Dissemination

Impingement
Temperature change
Shear

Rapid Transients

Evaporation
Solvent migration
Influence of gas phase

Delayed Adjustments (Pseudo-Equilibrium)

Solute migration
Structural rearrangements

Uptake by Host

Rehydration
Osmotic shock
Metabolic recovery
Temperature change

The starting point for our work was in the intriguing discovery of Monk* and co-workers that a particular microorganism dehydrated under particular conditions appeared to die extremely rapidly when exposed to a certain relative humidity around 94% and very much less rapidly at both higher and lower relative humidities. The idea that was generated by these findings was that of a "critical water content"—an unhappy way of putting it, perhaps, since the water present at the "critical water content" is obviously also present in the normal fully hydrated cell, so that it cannot be the water itself that is producing the lethal effects, but rather the absence of the water that was removed in attaining the "critical water content." The important conclusions to be drawn from Monk's work are, first, that withdrawal of a certain fraction of the cell water sets in motion certain processes that eventually have a lethal outcome. Second, that these lethal processes cannot be instantaneous or all-or-none affairs, because removal of even more water either slows them down or permits competing nonlethal reactions to take place. The final and equally significant conclusion to be drawn is that since these observations force us to indulge in comparisons, such as "slower" or "faster," we can scarcely hope to learn much about the mechanisms of loss of cell viability without getting involved in physicochemical and biological kinetics, and there is not much point in proceeding unless these qualitative distinctions can be solidly grounded.

* G.W. Monk. Personal communication.

II. WHAT ARE THE REQUIREMENTS FOR A SIMPLE QUANTITATIVE PREDICTION OF DECAY RATES OF DEHYDRATED ORGANISMS?

A. INTRODUCTION

During the course of our work on humidity effects we have arrived at opinions about the way in which kinetic data should be obtained and manipulated, and using only very simple mathematics have arrived at some decay equations that illustrate our point of view, even though they will undoubtedly turn out to be too primitive, and perhaps even wrong, when we are confronted with adequate experimental data. I would like to run over this development before going on to discuss experimental results, because I think it quite important in designing experiments to have a model to refer to, and one that offers some promise of leading to the genuine identification of mechanisms, rather than merely giving values of one or two constants (or more frequently non-constants) that can be interpreted only by taking refuge in such vague concepts as population heterogeneity or intrinsic probability.

B. SHORTCOMINGS OF FIRST-ORDER KINETICS

Like most of our colleagues, we have committed the fault of forcing reluctant data into a first-order pattern, largely because it feels more comfortable to be able to give a number instead of simply saying "faster" or "slower." This is only one of the serious objections to the uncritical use of first-order kinetics; equally serious is the fact that, when applied to finite populations rather than to the continuously varying concentrations of the physical chemist, first-order rate constants may lead to quite false predictions of the time taken, for instance, to achieve complete sterility. Sooner or later, after a more or less predictable number of "half-lives," the population will be down to a single viable organism, and then, after a less exactly predictable but nevertheless reasonable time, that too will die or disintegrate and the system will be sterile. One thinks of polio infections from supposedly killed virus, and in the context of airborne infection of whatever origin the behavior of residual viable populations of extremely low density is surely of considerable importance. We cannot get far with first-order processes or combinations of first-order processes without bringing in the idea that the surviving organisms represent a particularly resistant fraction of the population—an attractive hypothesis because of its ability to explain almost anything, but a dangerous one because it is so very refractory to all attempts at experimental verification. Otto Landman* once did an experiment in which he collected the survivors from a large population of aerosolized cells inactivated by

* Otto E. Landman. Personal communication.

desiccation, and he was surprised when I predicted correctly (after the experiment had been done) that the surviving cells, again aerosolized under the same conditions, would behave in exactly the same manner as the initial population, instead of being able to come through unscathed. No doubt many other examples could be found in the literature; I seem to recall one case in which investigators collecting cells that had survived exposure to an antibiotic were astonished and frustrated to find that it was impossible to distinguish these cells biochemically or in any other way from the original population. It is, of course, manifestly true that cell populations are heterogeneous; the hazard is in using this too readily as a crutch, instead of as a last-ditch position to take when everything else has failed.

C. THE NATURE OF THE DEVIATIONS FROM FIRST-ORDER KINETICS MOST COMMONLY ENCOUNTERED EXPERIMENTALLY

When a decay process is measured and when the logarithm of the number of surviving organisms is plotted against time, it is not unusual to find a number of points through which a reasonably straight line can be drawn. Such a line, however, seldom extrapolates to the correct starting point; it may suggest the starting population to have been either greater, or less, than measurements showed it to be (Fig. 1). At the other end, after longer periods of exposure, the line not infrequently flattens out, if one is able to take enough reliable measurements. Sometimes one can get back to the correct starting population more or less convincingly by drawing two intersecting straight lines, and sometimes a third straight line will take care of the flattening off after longer periods of exposure. However, the data are seldom good enough for one to be even reasonably certain that two intersecting straight lines would not be better represented by a single curve, and there are difficulties in interpreting such intersecting straight lines in terms of two or more first-order constants, unless one assumes that these apply to separate and independent portions of the population.

D. THE NATURE AND IMPORTANCE OF RELAXATION PROCESSES

If we restrain our enthusiasm for straight lines, and do our best to draw continuous curves through experimental decay data, we can easily see what sort of behavior will be shown instead of the lines in Figure 1. It is not difficult to think of quantitative explanations for such curves. If the reaction starts slowly and then speeds up, it may be that there is a reactant that takes a certain amount of time to reach the site of action or to accumulate; if an initially rapid action levels off, it could be because it is a self-limiting process as a result, for example, of the disappearance of something essential to the lethal reaction. If the curve does not level off completely, but continues to slope downward at a reduced rate, it might be that the exhaustion of the first rapid decay mechanism has made it possible for some second and much slower mechanism to show through.

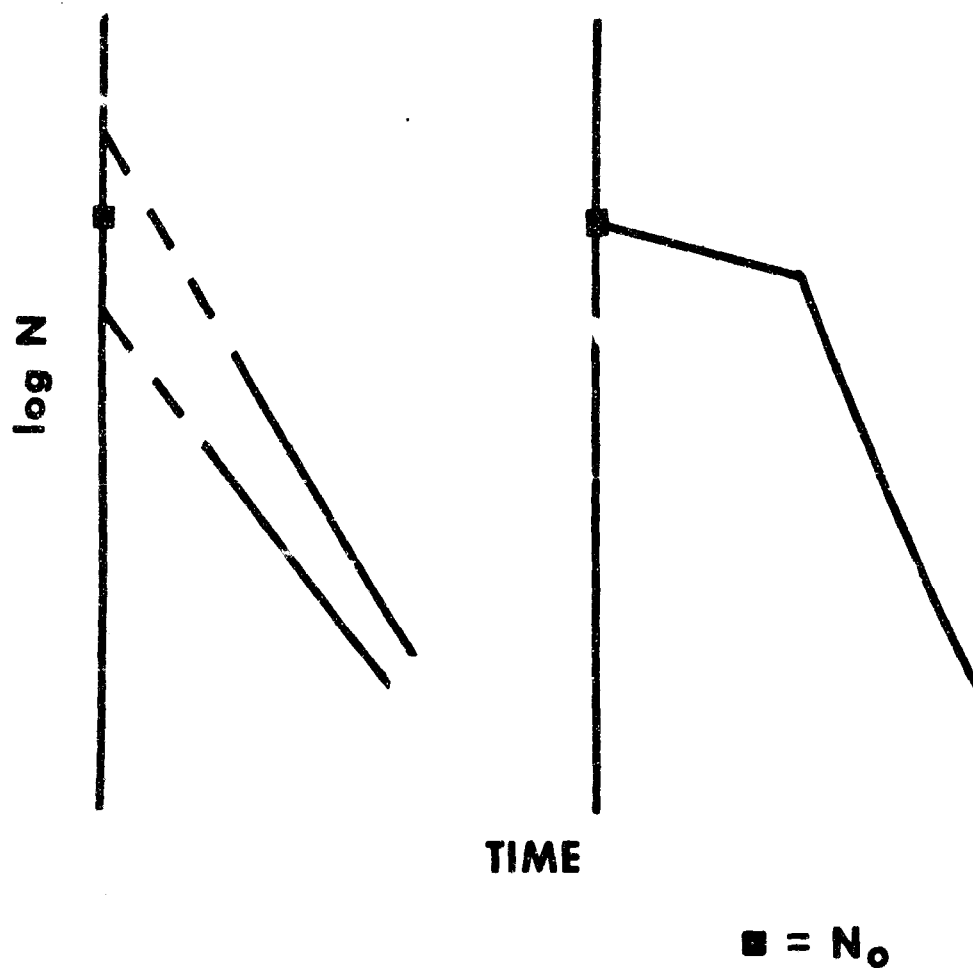


Figure 1. Kinds of Data Frequently Interpreted in Terms of Apparent First-Order Reaction Constants. Abscissa: time. Ordinate: $\log N$, where N is number of unreacted entities.

In all this we have spoken vaguely of processes, reactions, changes, and so forth. The important idea underlying this is that the kinetics of loss of viability must be determined by processes occurring at definite rates, of whose detailed nature we may be, and usually are, ignorant. An all-inclusive descriptive term for these processes is "relaxation," which has a certain mathematical precision while leaving detailed mechanisms unspecified. When a cell is subjected to some environmental change, its structures, solutes, chains, radicals, electric charges, and all the rest are thrown off balance, and each in its way swings to a new position of equilibrium. Giving the term relaxation the most general possible meaning, this will take care of everything that happens when a cell is subjected to a change in its environment, and each individual process can be assigned a relaxation time or a rate constant, at least in the context of the present attempt to set up a model for biological decay. Among all these processes, there must be some that are more significant for survival than others, and we make the simple assumption that for each type of lethal process there are only two such relaxations. In order to avoid the idea of heterogeneity within the cell population, we make the assumption that in each cell there are a sufficient number of representatives from each type of process for it to be represented by a first-order equation. If we consider only one such process, and think of each cell as a little test tube in which the identical process is taking place, and if approach to within a certain critical distance of equilibrium for this particular process defines the lethal condition, the entire population will die almost simultaneously after elapse of a certain time related to the relaxation time for the process in question. If instead we assume that the lethal condition is dependent upon two simultaneous relaxation processes as shown in Figure 2, then in place of the certainty of death when a certain stage is attained, we can postulate a probability dependent on the product of the two states of relaxation, thereby assuming that random competing events eliminate the inevitability of the lethal condition and permit a gradual decrease in the viable population. If, furthermore, one of the two relaxation processes leads to the complete elimination of one reactant, as in Figure 3, then the decay process will be self-limiting, and those cells that managed to escape death will be immortal, at least as far as this particular mechanism is concerned. Their chance of survival thereafter will depend upon the number of other lethal processes that may occur in the system, and upon their time constants.

E. SIMPLE EQUATIONS DERIVED FROM INTERACTION OF TWO FIRST-ORDER PROCESSES

Following the procedure just sketched, one arrives at a decay equation; it is unnecessary to bother you here either with the equation itself or with its derivation. The equation is really very simple. If we call the ratio of the initial population, N_0 , to the surviving population, N , the decay ratio, N_0/N , then the equation shows that instead of proceeding toward infinity as the reaction progresses, the decay ratio levels off

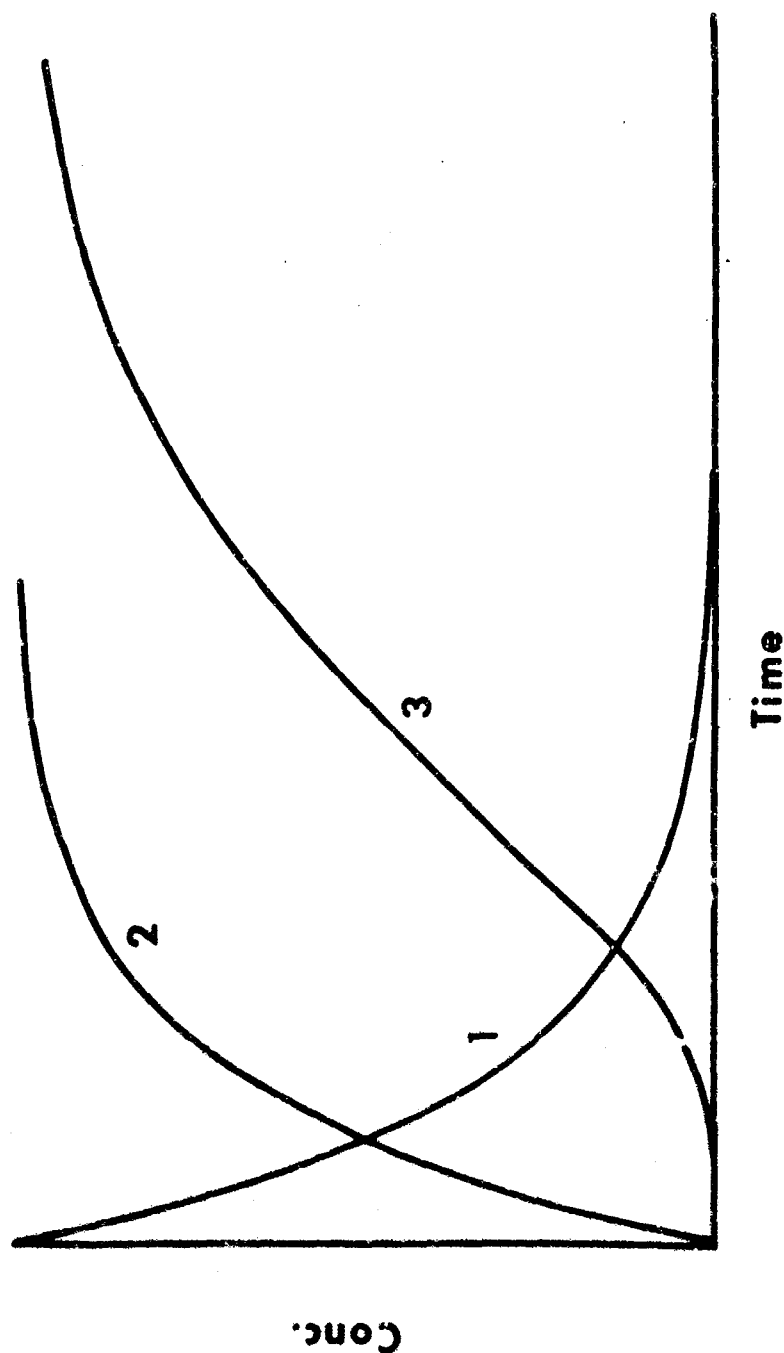


Figure 2. Processes Involved in Lethal Interactions. Abscissa: time. Ordinate: distance from equilibrium condition. Descending curve shows exponential die-away of one of the postulated reactants. Ascending curve 2 shows exponential accumulation of a postulated reactant or state. Ascending curve 3 shows sigmoid accumulation process. It is postulated that the lethal intracellular event results from the interaction of the descending component with one of the ascending components, the probability of death being proportional to the product of the respective "concentrations." Lethal reactions cease when the die-away of curve 1 is completed.

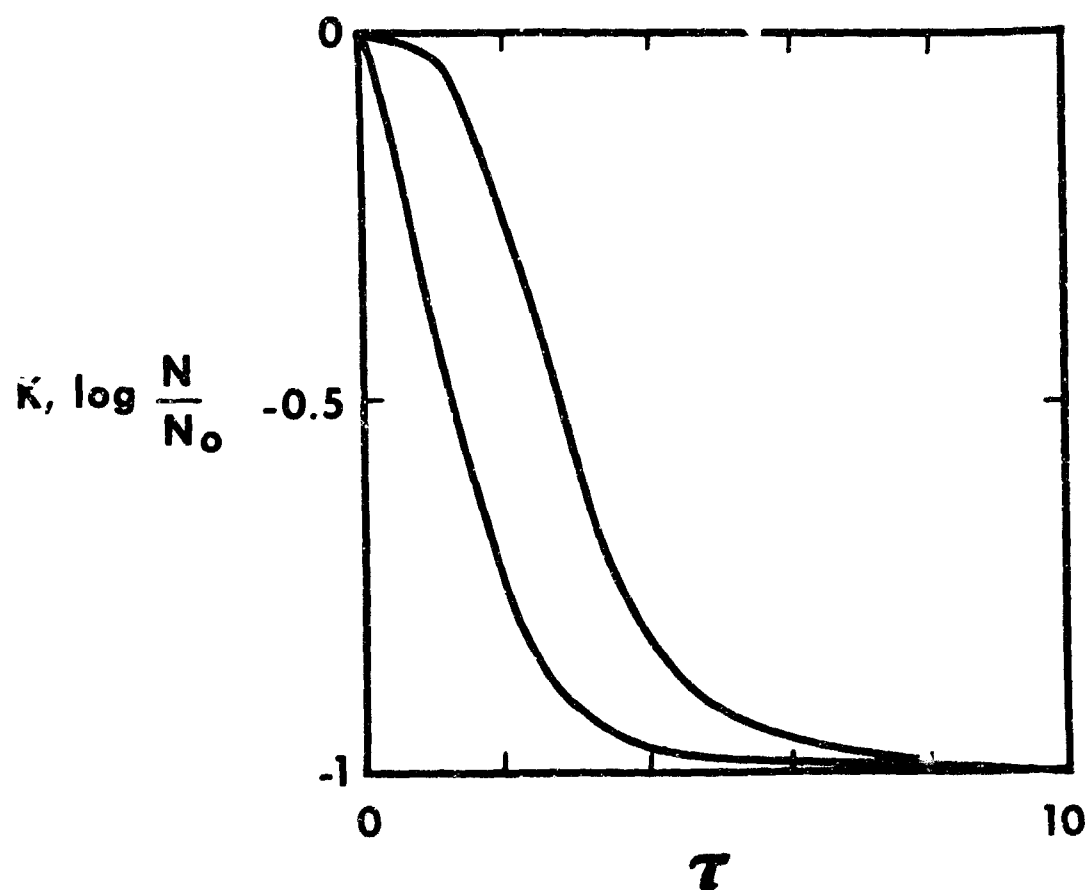


Figure . . . Examples of Calculated Decay Curves Plotted in Conventional Manner. Abcissa: a linear function of exposure time. Ordinate: a multiple of logarithm of the surviving or unreacted fraction of the initial material. Left curve: both postulated relaxation processes exponential. Right curve: exponential die-away process interacting with sigmoid accumulation.

to a constant value. Its logarithm, instead of being represented by a constant multiplied by elapsed time, as in a first-order process, is equal to the logarithm of this limiting value diminished by a term containing two negative exponentials of time. An illustration is given in Figures 3 and 4. The equation contains essentially three constants when arranged in its most simple form, the three constants themselves being simple functions of the time constants for the reactions concerned. With this modest number of parameters it should be by no means impossible to test the validity of an equation of this form and to derive values of the three parameters. One notable feature is that even this equation predicts a certain degree of quasi-first-order behavior over a more or less limited range, and indeed, because the limiting value of the decay ratio is determined by the time constants for the interacting processes, one can readily set up conditions under which the entire observable course of the process does appear to be of the first order; the leveling off would occur at such high values of the decay ratio that it would not ordinarily be detected before the material was virtually sterile. This is one of the obvious consequences of changing from infinite to finite populations; the range of values of the parameters for which experimental verification is feasible even in principle depends upon the absolute magnitude of the available initial population.

F. EQUATIONS LEADING TO AN INDUCTION PERIOD

It is easy to replace one of the exponential processes of Figure 2 by one having a sigmoid form, also shown in Figure 2, and, by going through the same procedure and making the same assumptions as to probability of a lethal outcome of the interaction, to arrive at an equation rather similar to the one just discussed but showing an induction period. Such curves are illustrated in Figures 3 and 5; after long periods of reaction they possess exactly the same features as those in the first equation.

G. DIFFUSION—LIMITED DECAY PROCESSES

The recent finding of DeWald* that some bacterial decay curves appear to have a linear dependence upon the square root of exposure time has led to the suggestion, by the same investigator, that the lethal event is diffusion-limited. This possibility is readily incorporated in the present hypothesis by postulating that upon removal of water from the bacterial cell a cell component, available in limited quantity, diffuses into another region of the cell, and that upon arrival there it is instantaneously converted to a lethal reaction product. The nature of a diffusion process that can continue for several hours at least within such a small volume remains a mystery. It is understood that a more sophisticated mathematical study is being undertaken by Lieutenant DeWald.

* R.R. DeWald. Unpublished observations.

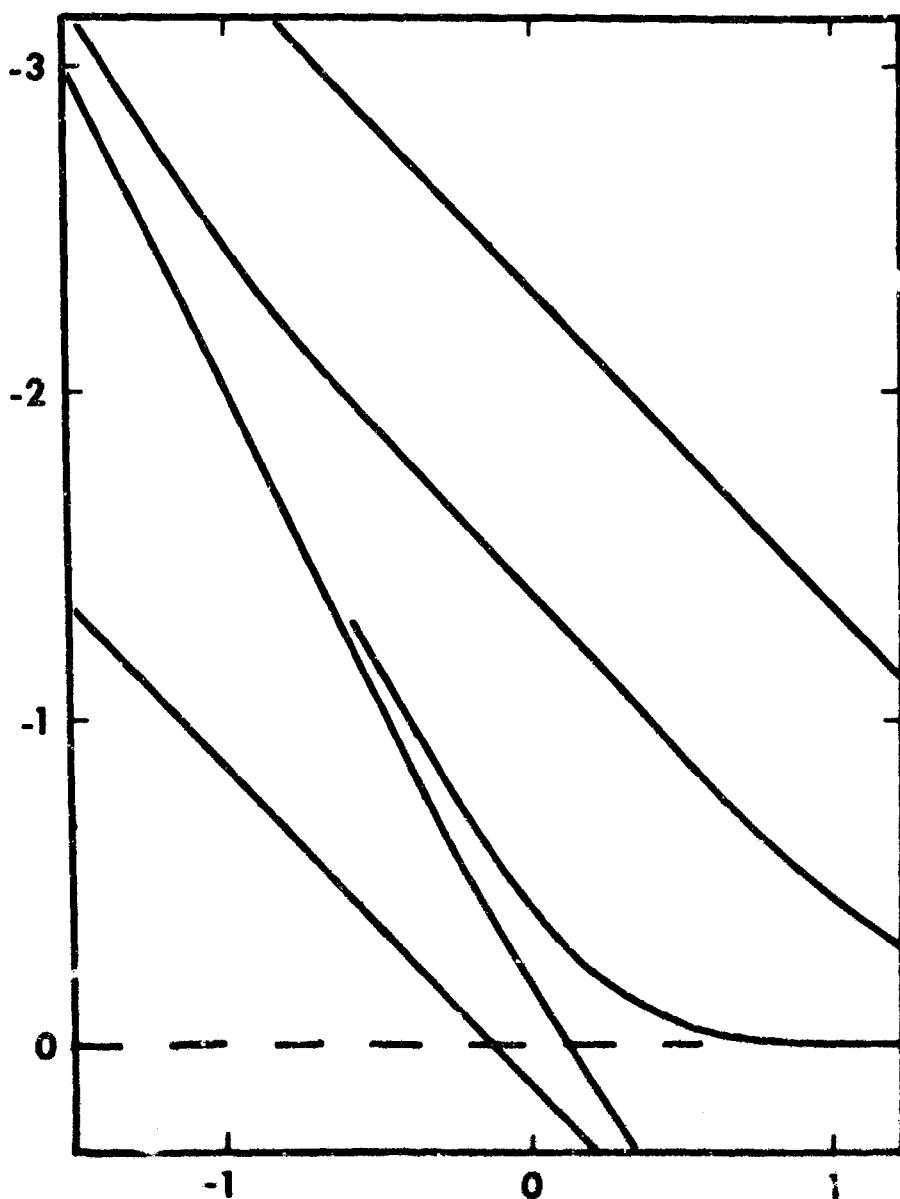


Figure 4. Some Theoretical Decay Curves Similar to the Left Curve of Figure 3 Plotted on Double Logarithmic Scales. Abcissae: logarithm of exposure time plus a function of the relaxation time constants. Ordinate: the double logarithm of the decay ratio (see text for definition) plus a function of the exponential relaxation time constants. The four upper curves are for different values of the ratios of the relaxation time constants; the line across the lower left corner represents first-order decay, with a slope of unity. The theoretical curves are characterized, for short exposure times, by a slope of two, changing under some conditions to a region of unit (or pseudo first-order) slope, and then leveling off. The fourth curve from the top is for the case in which there is no die-away of relaxation process one, so that the decay continues indefinitely, starting with slope two and changing to slope one.

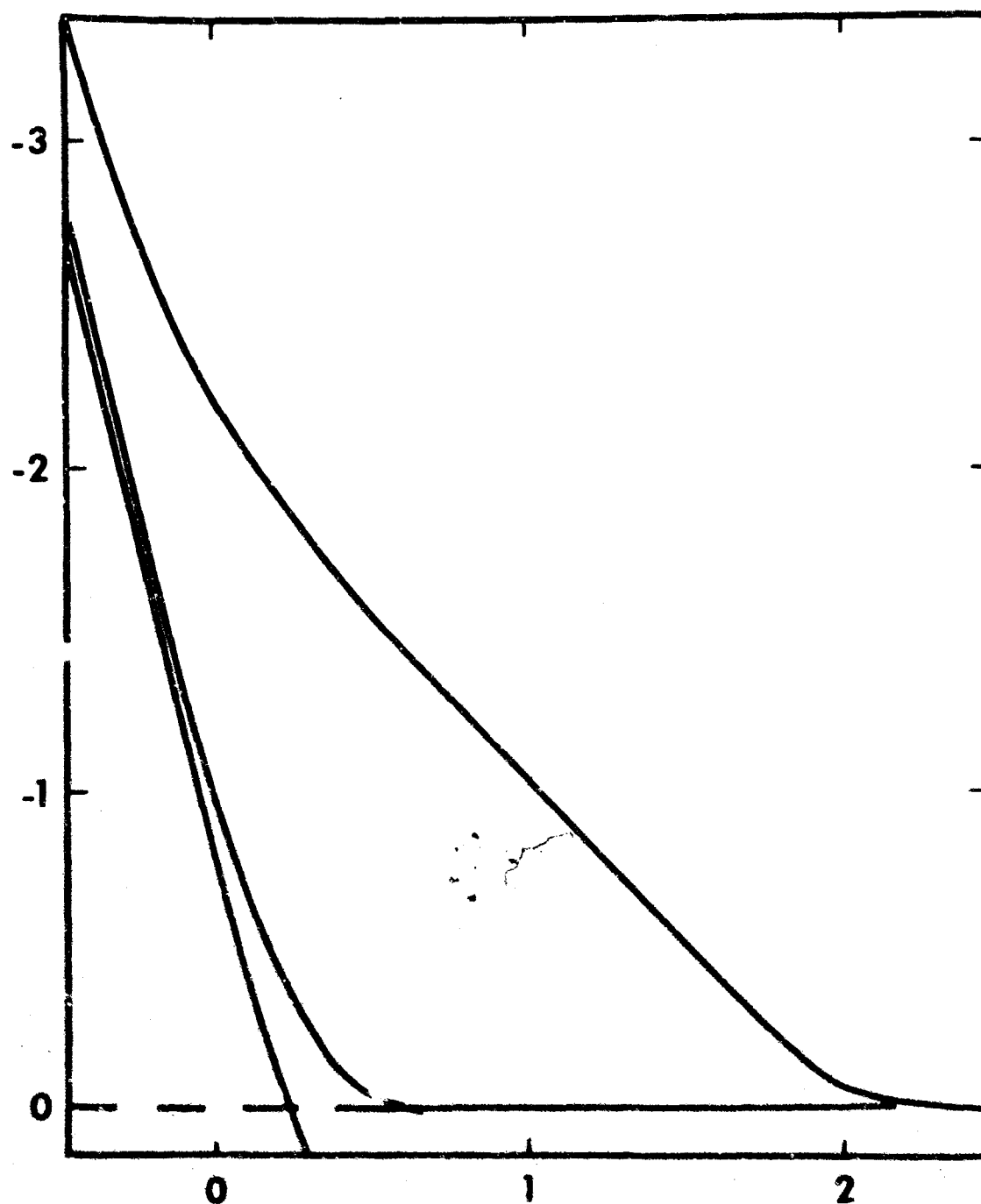


Figure 5. Double Logarithmic Plot of Theoretical Decay Curves for a Process with an Induction Period. These differ from the curves of Figure 4 in that the initial slope for short exposure times is five instead of two.

H. COMBINATION OF TWO OR MORE LETHAL INTERACTIONS

Given the multiplicity of cellular processes, one would think it unlikely that death would necessarily occur by only one route. One might indeed fear that the number of overlapping processes would make it quite impossible to unscramble them individually. It is experimentally unusual to see the clearcut establishment of a constant final viable population; more frequently, when there are signs of leveling off, one sees only a diminished rate of decay rather than a zero rate of decay, necessitating transfer to a new time scale in order to represent the results. It is easy enough in principle to combine as many lethal processes as one wishes, and to obtain equations that would undoubtedly be amenable to numerical manipulation by computer. Our ambitions have not extended beyond two sets of lethal interactions, but we have written the equations for these and have had a good many families of curves calculated by personnel of Biomathematics Division.

I. TREATMENT OF THEORETICAL CURVES FOR CONFRONTATION WITH EXPERIMENTAL DATA

Although our theoretical equations are by no means complicated, it would still be quite difficult to find numerical methods of solution. This would be true even in cases where only one process was involved, and it would become prohibitively difficult if two or more overlapping processes took place. Fortunately the equations are such that it is only necessary to present the experimental data graphically in somewhat unconventional form in order to be able to determine the values of the parameters, in principle, by a procedure of curve-matching by superposition. The experimental data are plotted with the logarithm of the exposure time as abscissa, against the double logarithm of the decay ratio as ordinate. The theoretical curves, on the other hand, are plotted in families having various sets of values of the parameters in such a way that the abscissa is equal to the logarithm of the exposure time plus a constant, while the ordinate is equal to the double logarithm of the decay ratio plus another constant; these constants are related to the time constants of the relaxation processes. Some examples of these theoretical curves are given in Figure 6. There are several interesting features, not all of which will be easy to identify experimentally. For instance, in cases where there is only one lethal interaction, the curves start off at short exposure times with a slope of two on the double logarithmic chart; as the exposure is prolonged, this may either eventually level off to a horizontal line indicating that the decay process is terminated, or may go through an intermediate stage in which there is a well-defined slope of unity, corresponding exactly to an apparent first-order process; this is then succeeded gradually by leveling off to a horizontal line. When there is an induction period at the beginning of the process, the initial slope, instead of being two, is five in the form of equation that we have been using. If there are two lethal processes, the curves are of course more complex, and whether the two processes can be clearly resolved as such by visual inspection

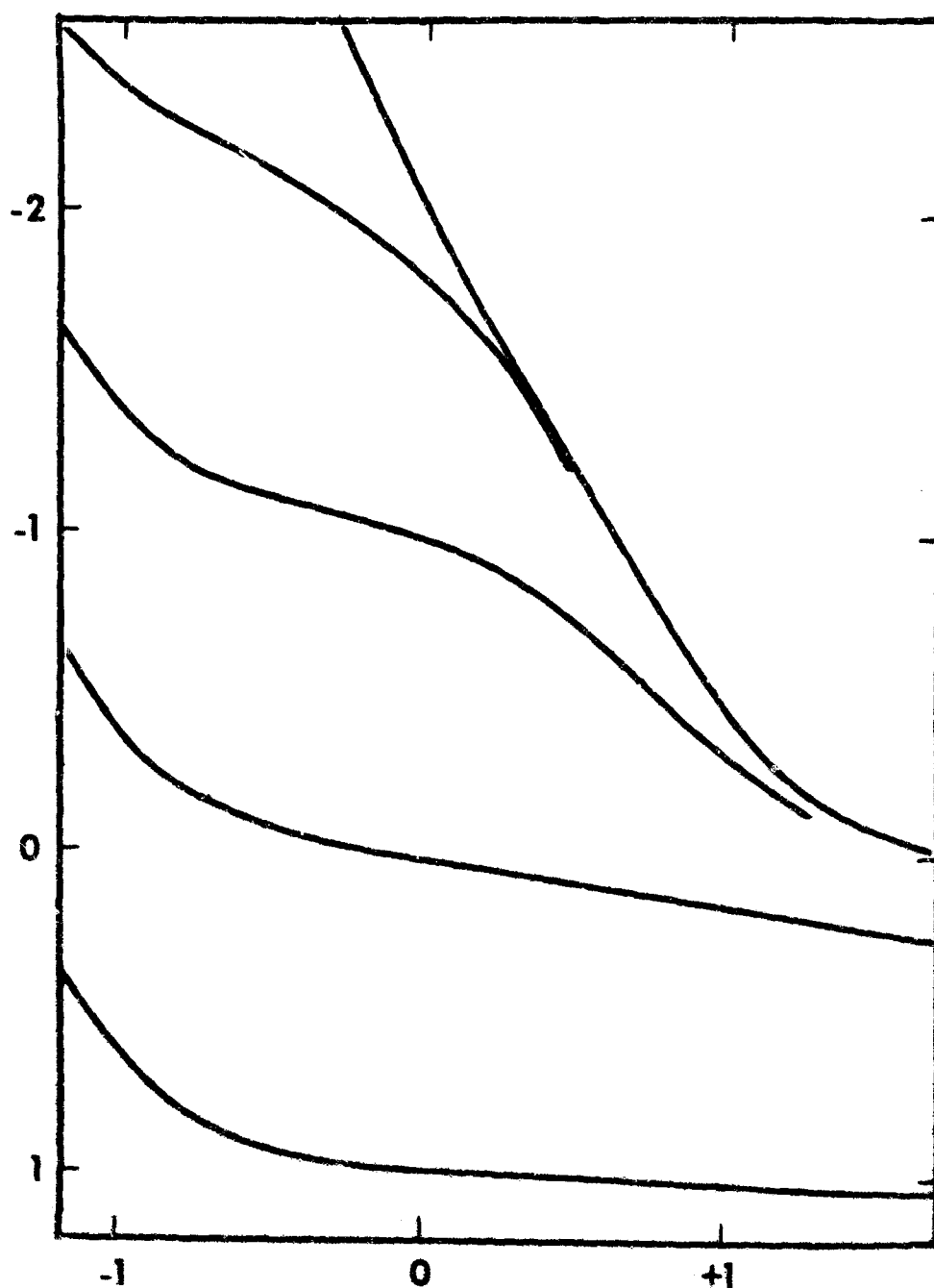


Figure 6. Examples of Theoretical Decay Curves Involving Two Sets of Lethal Interactions Occurring Simultaneously. Abscissa and ordinate are again respectively logarithm of exposure time and double logarithm of decay ratio, both displaced linearly by functions of the various relaxation time constants. Each curve approaches a different limiting value of the ordinate. In later calculations they have been further normalized so as to converge to a common limiting ordinate.

depends on how widely separated are their respective sets of time constants. If a reasonable degree of resolution occurs, then the first decay process appears to level off for some time, but at a later time the slope again increases to that characteristic of the main portion of the second lethal process, which by this time is taking care of the major fraction of the remaining population. Then this in turn levels off to a constant value.

The use of double logarithmic plotting of data is often thought to be a questionable procedure because of the reassuring way in which it irones out rather large irregularities. However, in the present context, it seems to have several decisive advantages. The first is of course the possibility of determining the parameters of experimental decay curves simply by a matching procedure, although it may turn out in practice that multiple solutions become rather a nuisance. Another point is that unless the experimental results are manifestly completely incompatible with this type of decay curve, which seems an unlikely eventuality, any decisive comparison of experimental data with theory will require sets of experimental observations taken over the most extended time period that may be feasible. This means observation over several logarithmic cycles on the time axis, and if graphical methods are to be used, the logarithm (or some function with similar properties) would seem to be unavoidable. Finally, variability in biological measurements being what it is, we should consider ourselves fortunate if we can obtain data to which any curves can be fitted without ambiguity even when the double logarithmic scale is employed.

III. THE EXPERIMENTAL APPROACH TO THE STUDY OF DECAY PROCESSES

A. INTRODUCTION

A modern biologist might well adopt as his motto "The Proper Study of Mankind is Mice." The opinion underlying our work on humidity effects is that the proper study of aerosols is almost anything but aerosols. For the good reason that irrelevant processes in aerosols are virtually uncontrollable, while their preparation and sampling both involve stresses that may be almost as serious as those nominally under investigation, we have looked for systems, without complete success, indeed, in which the effects of the dehydration stress can be studied without interference. The ideal requirements are easily stated: an assembly of a suitable number of separate and independent identical biological units, brought instantaneously to a quasi-equilibrium with an environment precisely defined as to chemical composition, humidity, and temperature, and after a suitable period of exposure instantaneously brought back to a condition under which a precise and unambiguous biological property can be measured with precision.

B. PREPARATION OF DEHYDRATED CELLS: MEMBRANE FILTER TECHNIQUE

Monk tried to get away from aerosols by using the sort of technique that a physicist or physical chemist might use on a simpler kind of system. He used large masses of cells, lyophilized and then exposed them to presumably constant relative humidity while still in vacuo. For a while we continued to use similar techniques, and in particular were able to show that the so-called critical water phenomenon was a great deal less striking when the cells were kept in air instead of in a vacuum, and also that at very low humidities there was a very pronounced transient effect of air that caused the recoveries to be much lower than they had been in vacuo. However, the reproducibility in such experiments is atrocious, and we soon came to the conclusion that this kind of technique, using massed cells instead of aerosols, is like jumping from the frying pan into the fire, if one can use such imagery in talking of freeze-dried materials. We made a good many attempts to get away from these difficulties, including a study by T. Green* of minute drops on pinpoints, but the most satisfactory solution always seemed to be to find a solid carrier upon which single cells could be uniformly distributed. One method investigated was to incorporate the cells in a monomolecular film of protein and to transfer them to a solid carrier by dipping a slide through the interface; such preparations had many advantages, especially for electron microscopy, but the mortality was considerable. No doubt a careful study of the reasons for this instability would be rewarding. The requirement of "instantaneous" equilibration with the ambient relative humidity is fundamental; and the water that delays equilibration most is the ordinary "wet" water in a cell suspension (Fig. 7). It is imperative therefore to find some way of blotting the cells as soon as they are deposited. For a number of years now we have been doing this by placing a very small volume (0.02 ml) of the cell suspension upon a membrane filter to which is attached, on the underside, one of the absorbent pads that the manufacturer used to supply with the filters, and which he now sells separately. As soon as the filter looks dry, in a matter of a second or two, it is stripped off the backing and placed in a suitable vessel containing a saturated solution of defined relative humidity, and this vessel in turn, in the most careful work, is placed on a shaker and immersed in a constant temperature bath.

C. BIOLOGICAL CRITERIA OF INJURY; THE QUESTION OF RECONSTITUTION AND ASSOCIATED LOSSES

Usually a number of these membrane filter preparations are placed in separate vessels at the same time, and are withdrawn after various times of exposure to the chosen relative humidity. The cells are immediately eluted with water or nutrient broth and suitable solutions are made for plating and incubation. Of course this is a very primitive method of

* T.D. Green. Personal communication.

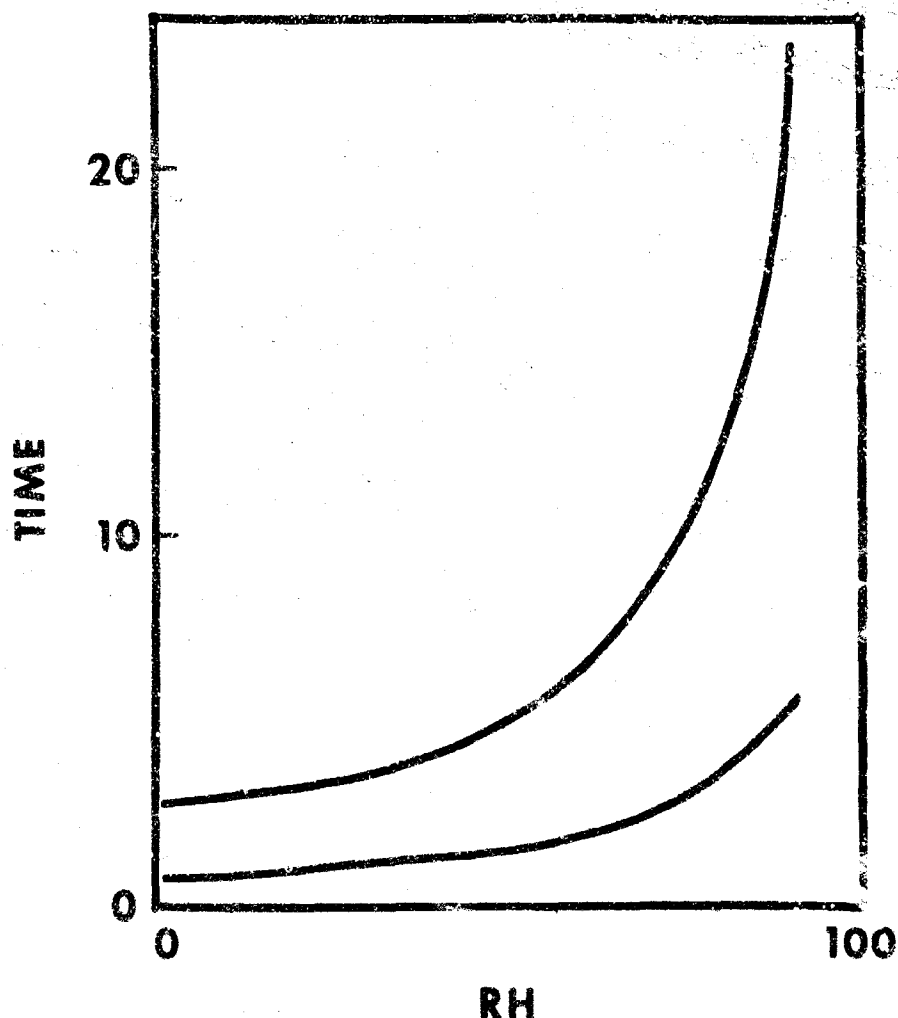


Figure 7. Relative Equilibration Times for Bacteria Exposed to Different Relative Humidities. Abscissa: final relative humidity. Ordinate: relative time for 90% equilibration with final humidity. The curves are based upon experimental water absorption isotherms for bacteria, together with simple assumptions as to the relationship between rate of evaporation and vapor pressure differential. The upper curve applies to fully hydrated cells with an initial water content 2.4 grams per gram dry weight. The lower curve refers to cells that have first been dehydrated to the upper level of the adsorption isotherm, corresponding to an equilibrium relative humidity in the vicinity of 90%, the initial water content in this case being 0.63 gram per gram dry weight. It is seen that if large differential rates of evaporation at different relative humidities are to be avoided, a preliminary equilibration for removal of most of the ordinary intercellular water would be desirable.

detecting injury; it is not always the all-or-none phenomenon that one would wish it to be, and there is always the question of secondary losses caused by the reconstitution procedure itself. One has to be on the lookout for osmotic shock effects, which we have found particularly troublesome in the case of preparations containing glycerol, although strangely enough they do not seem to occur in the case of urea. One also has to be alert to any abnormality in the colonies or in the time of their appearance; in preparations containing urea, for example, both in bulk suspension and in membrane filter preparations we have always found that the colonies are extremely variable in size and that there is considerable delay in their first appearance on the plate. It is interesting to ask whether cells that do not divide within the normal interval after plating ought to be considered dead from the point of view of tests of this kind, the presumption being that if they do not behave normally they must have suffered the same injury as dead cells, with the difference that there is some sort of recovery mechanism that enables them to divide later on. Obviously there is a need for the study of various possible criteria of injury, and our work certainly has suffered from the shortcoming that it has dealt only with viable counts done by conventional methods.

D. CONTROL OF THE ENVIRONMENT

In a considerable number of cases the relative humidity in equilibrium with a saturated aqueous solution of an inorganic substance is known, and such solutions provide a convenient means of establishing the desired environment with respect to the partial pressure of water vapor. The properties of these solutions are fairly well documented physicochemically, at some single temperature in most cases, but independent check of some of these values is certainly to be desired and will perhaps prove possible in our laboratory by vapor pressure methods. One also has to be aware of the possibility that traces of volatile decomposition products or contaminants may produce some of the biological effects observed, or that the solution may modify the carbon dioxide partial pressure in the atmosphere of the experimental vessel. Indeed, the partial pressure of carbon dioxide is a factor of possible significance in the variability of our data and one that has not hitherto been controlled. In the experiments that we are about to begin we are planning to replace these constant-humidity solutions by a stream of gas previously moistened to the desired degree. When very low humidities are needed, we expect this technique to be quite successful; at higher humidities precise equilibration of the gas stream may prove to be rather difficult.

E. PREPARATION OF UNIFORM TEST MATERIALS

Our stipulation that the biological preparation should consist of a suitable number of identical biological entities is of course rather fanciful. In any ordinary method of preparation the cells certainly exist in various states of development and some are dead to begin with.

We have worked mainly, more or less by force of habit, with 18-hour cultures from agar slants in Roux bottles, suspended in water and twice-washed in distilled water. It would be better to use liquid cultures, possibly continuous cultures, although this would by no means necessarily lead to more uniform material, and perhaps synthetic media should be used. We have done only enough along these lines to have become aware that cells prepared by these methods show significantly different behavior in the humidity chambers from those grown on agar. If synchronous growth were a more reliable and reproducible phenomenon, synchronized cultures would of course be very desirable.

IV. GENERAL APPROACHES TO THE RECOGNITION AND IDENTIFICATION OF LETHAL RELAXATION INTERACTIONS

A. DEPENDENCE OF DECAY RATES UPON RELATIVE HUMIDITY UNDER OTHERWISE CONSTANT CONDITIONS

Relative humidity as such seems to control the mortality rate of bacterial cells. Whether or not there are actually maxima or minima in decay rates as a function of relative humidity, any physical picture of the drying process, involving successive uncovering of different types of chemical groupings as the humidity is decreased, points almost indisputably to the existence of more than one decay mechanism as long as the decay rate and the decay pattern vary with changes of humidity. A single mechanism, associated perhaps with the uncovering of specific sets of chemical grouping, would lead one to expect cells to be quite stable over a certain range of relative humidities, and then uniformly unstable at relative humidities below a threshold value. While there is no evidence that such simple behavior will be encountered, it is clearly relevant to the whole problem of lethal interactions to have the most complete information possible upon the decay rate as a function of relative humidity, at least for one organism and one specified set of conditions. In view of the theoretical approach outlined above, it may be misleading to speak of "decay rates" where one obviously must consider the entire pattern of decay as a function of time and its compatibility with some kind of theoretical formulation.

B. THE USE OF PHYSICOCHEMICALLY WELL-DEFINED ADDITIVES

Important clues as to the lethal interactions ought to be obtainable by using simple and biologically nonspecific substances and applying a little quite elementary physical chemistry. The concentration of salt in a drop of sodium chloride solution exposed to reduced relative humidity, for instance, will always have a perfectly definite equilibrium value; and at some perfectly definite threshold value of the relative humidity it

will dry up completely and at all lower relative humidities will consist simply of a crystal of sodium chloride. If sodium chloride in ordinary solution produces some biological effect that is a function of concentration, this effect will be observable when cell suspensions containing salt are equilibrated at various relative humidities; and if the relative humidity is so low that the salt dries up and produces a crystal, then according to this simple description of what is going on it should cease to have any biological effect. There should in fact be a threshold for biological action. If this is not what we find in carrying out such experiments, then at once we have some evidence that this salt modifies the effect of relative humidity in some indirect way, for example by being attached to some part of the system and behaving therefore not like free salt, or by being supersaturated, or in some other way. Other illustrations of this type of experiment will be shown in the experimental part of this paper.

C. MODIFICATION OF DECAY CURVES BY CHANGING CONDITIONS IN A MANNER EXPECTED TO PRODUCE DIFFERENTIAL CHANGES IN RELAXATION TIMES

A traditional approach to the study of phenomena that are not very reproducible or that are not amenable to accurate absolute measurements is to look for changes, in the belief that these can be more accurately and reliably measured than the absolute quantities themselves. Because of the very large number of measurements needed, even under the best conditions, if we want to get an idea of the whole pattern of decay, it is not at all likely that this approach will be of very much value until our techniques and the reproducibility of our measurements have improved. However, even if a few experimental points only can be obtained, such comparative data may be rather informative, and might indeed provide basic evidence for or against the lethal interaction mechanism that we propose. If at least two processes are involved in the decay phenomenon, it is most unlikely that both will vary in the same manner under all conditions, and indeed the systematic variation of decay pattern with relative humidity may itself provide evidence that differential effects in a single set of interactions are involved, rather than the introduction of new sets. It might also be supposed that other relatively nonspecific physical influences, such as changes of temperature, would produce differential effects resulting in modification of the decay rates. This approach might be applied in several ways. For example, a preliminary exposure to a bacteriostatic temperature might perhaps result in excessive accumulation or depletion of one of the reactive substances or states, leading to radical changes in the response to a subsequent alteration of relative humidity. On the other hand, carrying out the exposures to lowered humidity at different temperatures might itself provide important clues to the manner in which the respective time constants are affected. As we gradually get a better idea of the precise interactions involved, it might well be that more specific influences such as light or ionizing radiation might also be useful tools in the study of humidity-dependent effects.

D. ATTEMPTS TO REDUCE THE NUMBER OF POSSIBLE OPERATIVE LETHAL INTERACTIONS IN A CELL SYSTEM

We have no conclusive proof that under any specified set of conditions several lethal pathways are important, but it is not too early to be thinking of this possibility, and also to give some consideration to the associated problem of repair processes. One of the more serious shortcomings of the viable count method of assessing injury is that it really may not always be an infallible index of a particular lethal interaction, since under some conditions the cell may be capable of instituting a bypass resulting in recovery when, from the limited point of view of the immediate experiment, it should be dead. There seems to be no obvious way of reducing the number of pathways by which a cell can die under given conditions, although an experienced microbiologist might be able to make some inspired guesses as to the most suitable choice of organism, of its physiological state, and its biochemical environment and make-up. Something might be done, for instance, with cells having well-defined biochemical deficiencies and grown in a minimal medium; but the principle of this choice is not altogether clear, for one might also argue that the richer the cell's resources, genetically and enzymatically, the fewer the possible lethal pathways.

E. EVALUATION OF DECAY PARAMETERS FROM DETAILED LONG-TERM DECAY CURVES FOR COMPARISON WITH THEORETICAL EQUATIONS

Mention has already been made of the graphic method by which it ought to be possible to calculate values of the decay parameters from experimental decay curves. There is a need for extended observation, starting from the earliest point at which gross equilibration is presumed to have been completed, up to periods of weeks.

F. BIOLOGICAL MODIFICATION OF CELLS TO PRODUCE DIFFERENTIAL CHANGES IN RELAXATION TIMES

We have speculated above upon the possibility of producing differential changes in time constants by nonspecific physical means. In the long run the study of the effects of biologically specific changes may of course prove more valuable and more informative. The number of such possible experiments, including the use of cells in different physiological states, the choice of various strains and mutants, the modification of the biochemical nature of cells by exposure to drugs, antibiotics, metabolites and so forth is enormous. One approach in which we have been particularly interested is the study of cells containing attached or intracellular bacteriophage at various stages of the replication cycle. Miss Mary Davis* started on this problem around 1957, attempting to do experiments of this

*Mary S. Davis. Personal communication.

sort with a bacteriophage for Serratia marcescens, since we felt that we had already a good deal of necessary information on the sensitivity of this particular host to humidity changes. This turned out to be a very unsatisfactory phage to work with, and when a year ago Mr. Abraham L. Turetsky became available for work of this sort, we decided to turn to a T3 coliphage, realizing that this would necessitate sooner or later an independent study of humidity effects upon Escherichia coli. We have had some preliminary success in depositing cell-phage complexes upon membrane filters and in observing the burst pattern by the Sinsheimer filtration technique, but incidental losses of cell viability and bacteriophage are serious and erratic, and a definitive technique has yet to be worked out.

G. CHOICE OF OTHER EXPERIMENTAL ORGANISMS

The possibility of choosing an organism more suitable for the study of decay processes in their simplest form has been touched on in Section IV, D. Other organisms, suitably chosen, have also a comparative interest if they are selected for the sake of peculiarities that may prove to be specifically coupled to their sensitivity to dehydration. Bacterial spores and halophilic, thermophilic, and psychrophilic bacteria come to mind; the viruses, which have been shown by Hammes to be humidity-sensitive, may prove to be of particular interest because of their presumed greater simplicity of structure and functional capability.

V. RESUME OF EXPERIMENTAL RESULTS TO DATE*

A. RELATIVE HUMIDITY AND DECAY OF WASHED CELLS

Our preliminary survey of the effects of diminished relative humidity upon washed *Serratia marcescens*, as soon as we were satisfied with the membrane filter technique, took quite a long time, but in retrospect it seems to have been regrettably limited to rather short exposure times, seldom in excess of 72 hours. Curves were obtained at some 13 different values of the relative humidity ranging from 98% to a nominal zero produced by solid potassium perchlorate. Most of the main features of these curves are shown in Figures 8 through 11. The actual curves plotted in Figures 8 and 9 for rather widely spaced values of the relative humidity show only the general trend, which, both for short and for more prolonged exposures, is in the direction of decreased stability with decreased relative humidity. They also illustrate a point mentioned previously, that if the observations are made over a sufficiently limited period of time it may very well look as though a first-order process is going to continue indefinitely, as in the data at zero humidity, or it may seem, as was apparent at 33% relative humidity, that the curve is leveling off and that no further important decay is likely to take place. Another point, not very clearly brought out in the diagrams, is a very annoying initial loss that seems to be connected more with the initial contact with the membrane filter than with a real humidity effect. We have been very much concerned with this because it introduces some uncertainty into the value of the initial viable count, which should be used in any calculation done with the data. We have given preliminary study to a way around this difficulty by holding the preparation for some time to settle down at a high humidity such as 98 or 99% before reducing the humidity to the desired value. This may have the advantage of giving us a definite initial count, but the disadvantage of perhaps slowing down the gross equilibration, since the membrane filter will have become saturated with water at a rather high humidity. In Figures 10 and 11 all our early data are summarized by the method that we have so sharply criticized in the earlier part of this paper, namely that of dividing the decay curves into linear logarithmic segments and assigning to them apparent decay constants in units of reciprocal hours. The summaries in Figures 10 and 11 should be regarded as having little

* The data in this section are presented in conventional manner by plotting the logarithm of the fraction of viable cells against time and by characterizing the curves obtained in terms of one or more numbers referred to as "decay constants." In preparing these data for more formal presentation it has been found preferable to plot the double logarithm of the "decay ratio" (see Section II, E) against the logarithm of elapsed time of exposure. Within the limits of experimental error the data so plotted can be represented in terms of one straight line or two intersecting straight lines in short-term experiments of the type reported in this section.

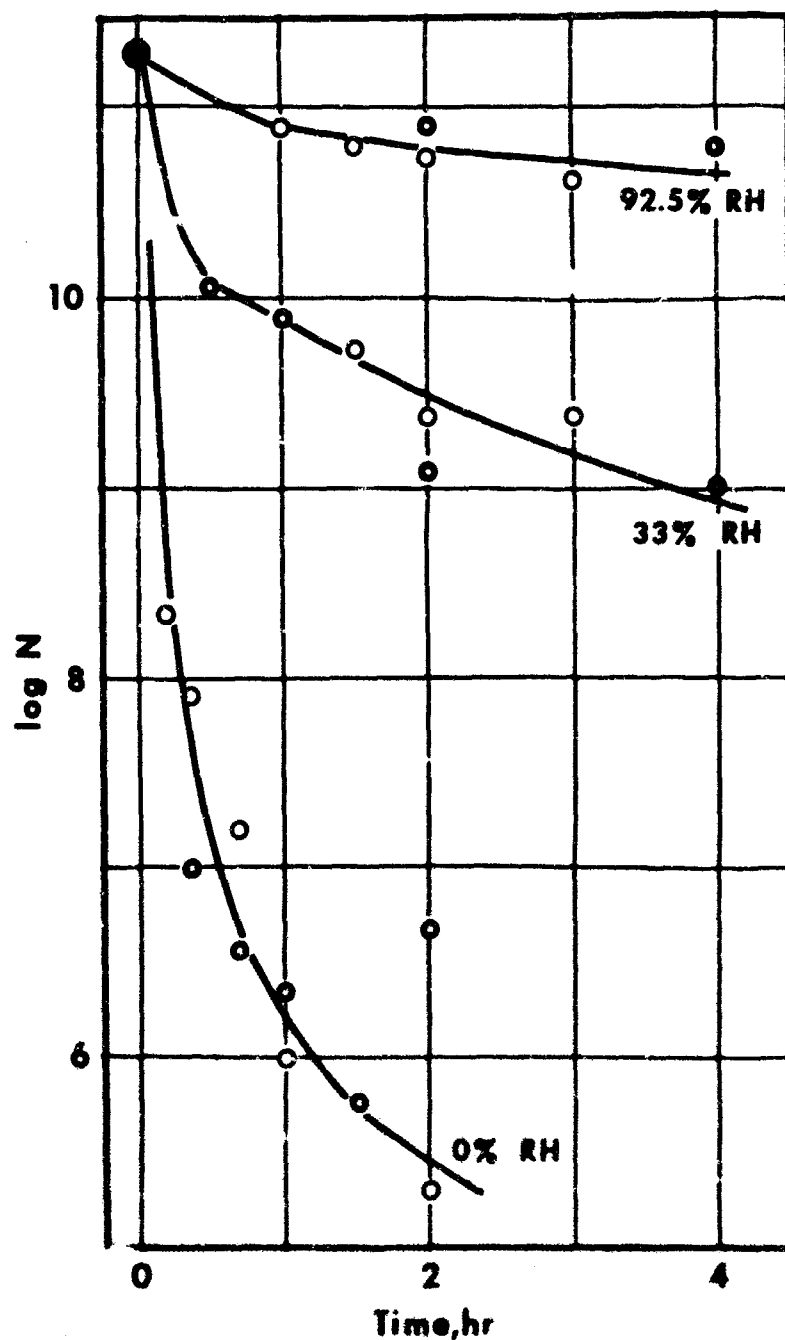


Figure 8. Representative Decay Curves for Washed *Serratia marcescens* 80K Obtained by the Membrane Filter Technique. Abscissa: exposure time in hours. Ordinate: logarithm of viable cell count per ml, referred back to the stock suspension. The actual number of cells used in each exposure is about 10^8 . In all cases the results of two separate experiments are presented.

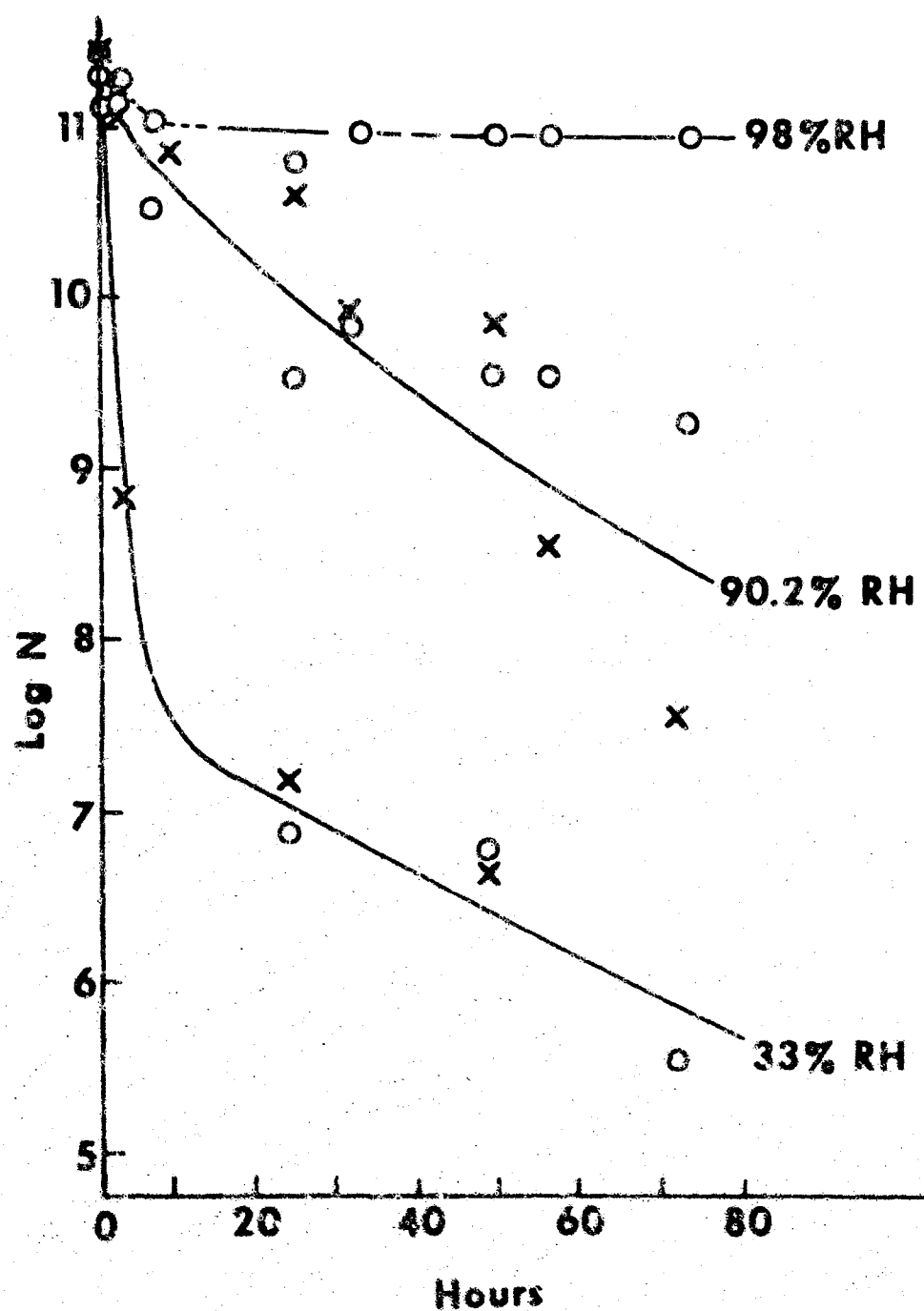


Figure 9. Decay Curves for Washed *Serratia marcescens*, Obtained by Membrane Filter Technique; Results of More Prolonged Exposures. Abscissa: exposure time in hours. Ordinate: logarithm of surviving cell count.

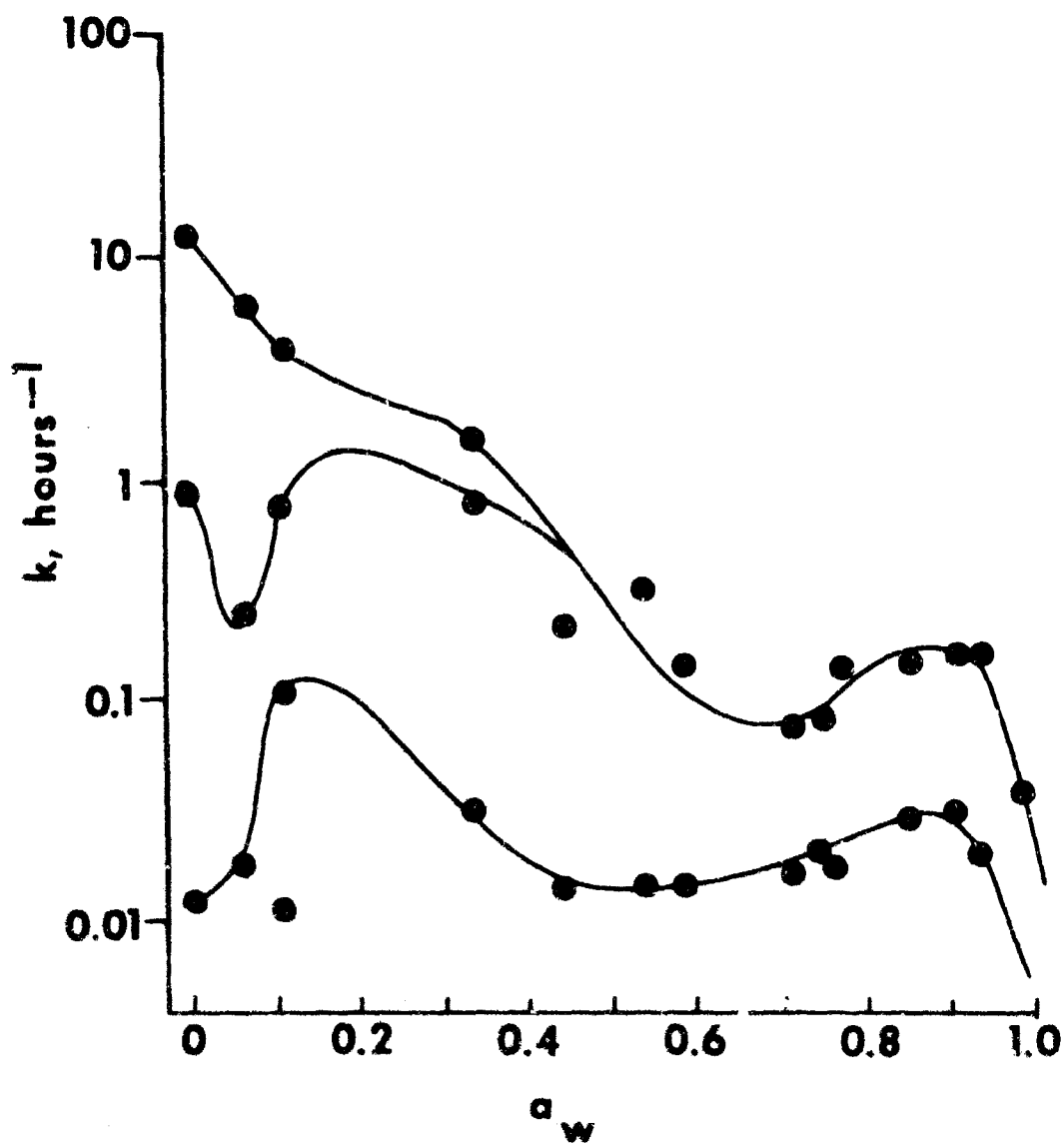


Figure 10. Summary of Results of Measuring Slopes of Approximately Linear Segments of Decay Curves for Washed *Serratia marcescens* at Different Relative Humidities. Abscissa: water activity, or relative humidity divided by one hundred. Ordinate: slopes of linear segments, or pseudo-first-order time constants, in reciprocal hours.

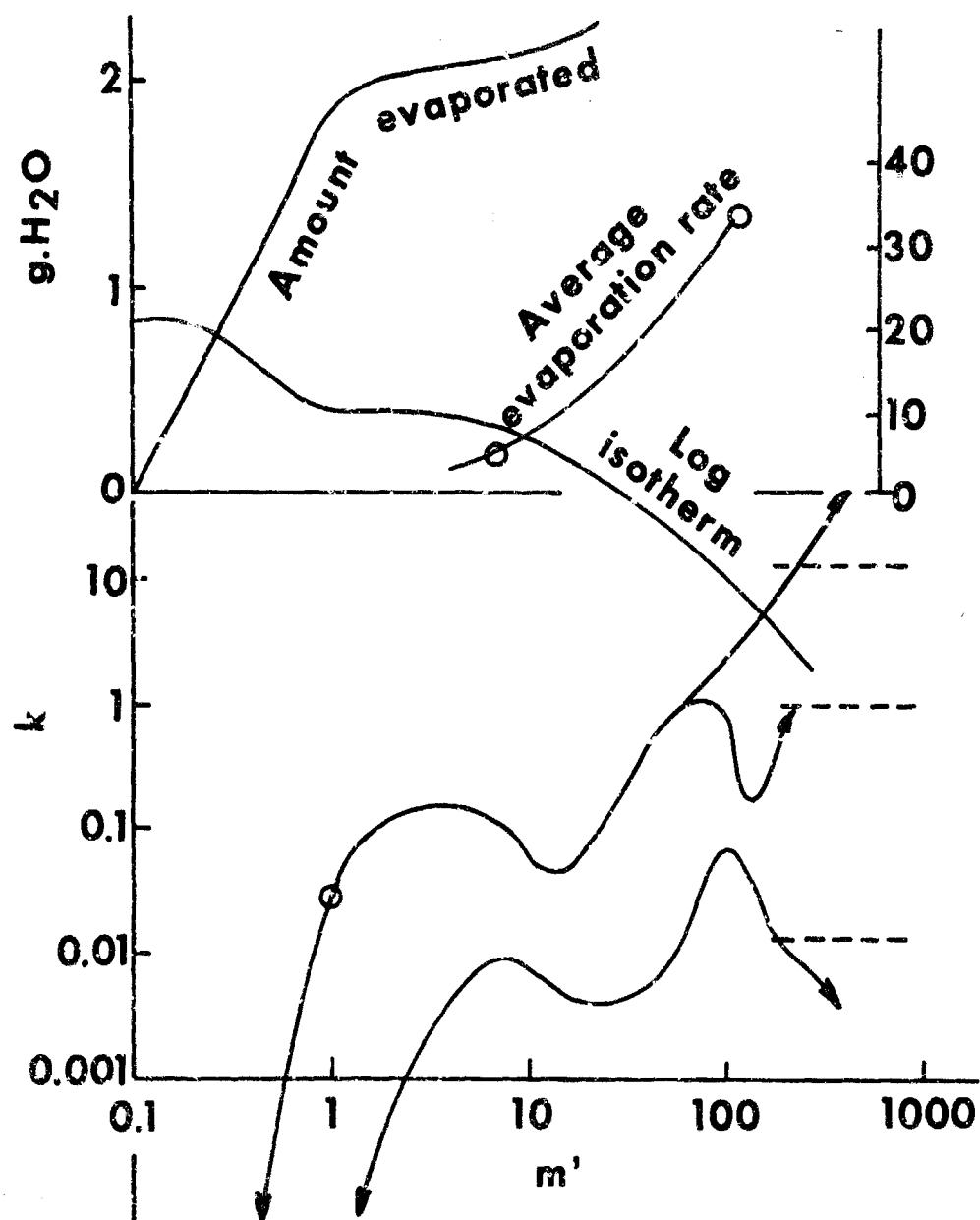


Figure 11. Data of Figure 10 Replotted for Comparison with Amounts of Water Lost and Average Evaporation Rate. Abscissa: effective external osmolality, which is related to the negative logarithm of the relative humidity. Lower ordinate: effective first-order constants as given in Figure 10. Upper left ordinate: amount of water that must be removed to bring cells into equilibrium at the specified humidity or at the external osmolality, in grams of water per gram of dry cells. Upper right ordinate: average evaporation rate in arbitrary units.

more than qualitative value, together with an indication of the fact that the kinetics involved are rather complicated, and that they are not the same at all values of the relative humidity. Indeed, at humidities below about 40% we have had to divide the decay curves into three parts, and each shows a distinctive trend as the humidity is further decreased. An extremely rapid component becomes more and more prominent all the way down to zero relative humidity, although here the slowest portion of the decay is actually somewhat slower than that found at humidities above 90%. This is simply a way of saying that although exposure to extremely low humidities results very quickly in loss of most of the cells, there is a far greater tendency than elsewhere for a small fraction to survive for a very long time. As a matter of fact, on a number of occasions we observed after prolonged exposure a very intriguing upward turn of the recovery figures, which we have the audacity to think of (but not publicly) in terms of a possible extremely slow recovery of viability at extreme desiccation. In Figure 11 the same decay "constants" are plotted with the amount of water that has to be removed from the cells in order to attain "equilibrium" with the ambient relative humidity. A very rough curve for the average evaporation rate during the process of equilibration is also included. The amount evaporated, of course, levels off after the gross bulk water has disappeared, but there is nothing in the curve to suggest what appear to be the minima in the decay rates. On the other hand, the rapid rise in initial decay rate in the lower humidity ranges might be correlated with a rather rapid rise in the average evaporation rate, suggesting perhaps that this portion of the decay has more to do with the transport of water than with any equilibrium or quasi-equilibrium configuration of exposed groupings.

B. EFFECTS OF SODIUM CHLORIDE, LITHIUM BROMIDE, AND UREA

The rationale of experiments using additives such as these has been explained in Section IV, B. The particular choice of sodium chloride, lithium bromide, and urea was dictated by the following properties: (1) sodium chloride and lithium bromide, although their biological effect is so, no means well defined, are interesting in that they are both alkali halides yet they differ radically in the properties of their saturated solutions. All the water evaporates from sodium chloride at humidities below about 75%, but lithium bromide remains in solution until the humidity reaches roughly 7% (Figures 12 and 13). According to the argument already given, therefore, if these substances produce their effects in a straightforward way related to their concentration in ordinary aqueous solution, lithium bromide should remain active down to very low relative humidities, and sodium chloride should have a cutoff at about 75%. It happens, as shown in Figure 14, that both these substances produce some added mortality at very high humidities, and it seems that the lethal effect of sodium chloride persists down to a relative humidity of 33%, a long way below its solubility limit. The

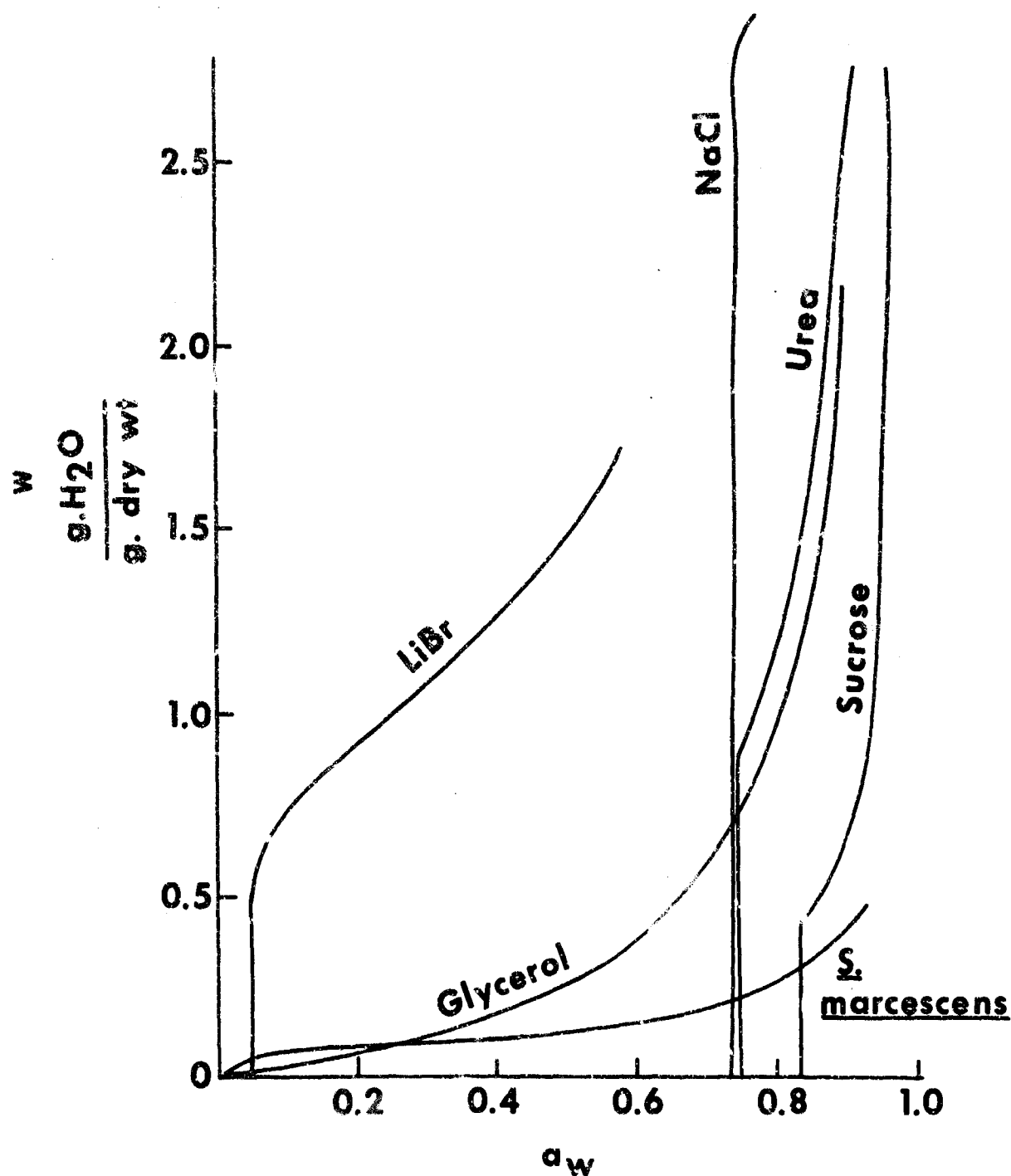


Figure 12. Water Contents of Various Simple Aqueous Solutions Equilibrated with Different Water Activities or Relative Humidities. Abscissa: water activity at equilibrium. Ordinate: water content in grams of water per gram of solute. The solubility limits or cutoff points are shown for sucrose, sodium chloride, lithium bromide, urea, and glycerol. The water adsorption curve for *S. marcescens* is also indicated.

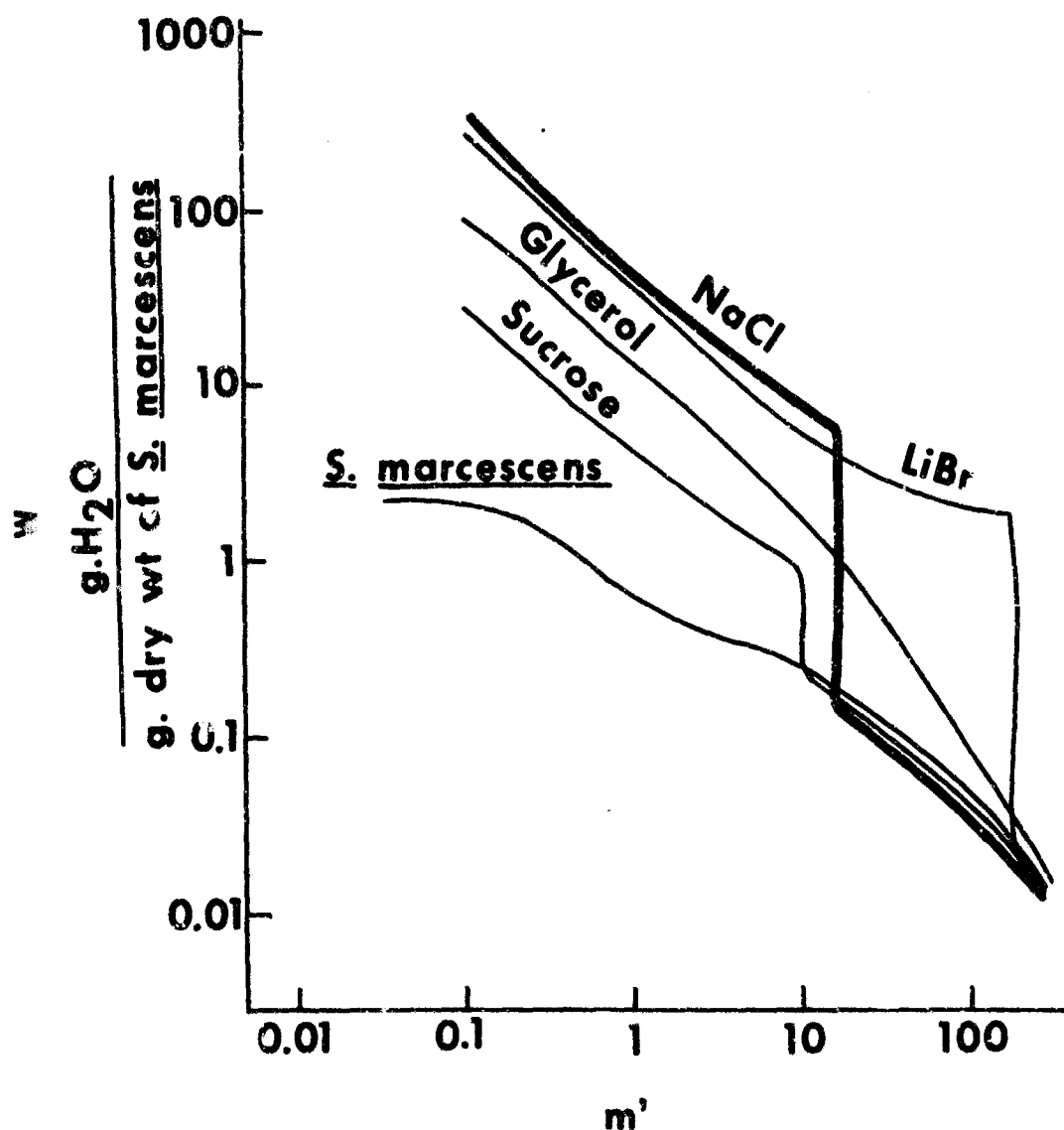


Figure 13. Data of Figure 12 Recalculated to Show the Variation with Relative Humidity of the Water Content of *S. marcescens* to Which Various Substances have been Added in the Constant Proportion of One Gram to One Gram of Dry Cells. Abcissa: osmolality (see Figure 11). Ordinate: grams of water at equilibrium per gram dry weight of *S. marcescens*. In Figures 12 and 13 the behavior below the cutoff points cannot be indicated precisely, because the water contributed by adsorption on the solutes depends upon the size of the crystals produced. If gross crystallization is prevented and aggregates comparable in size to the bacterial cells or smaller are formed, then the water attached to these solutes could be comparable in amount to that adsorbed by the bacteria.



Figure 14. Effects of Sodium Chloride and Lithium Bromide Upon Decay Curves for Washed *S. marcescens*, at Four Values of the Relative Humidity. The curves were determined by the membrane filter technique. Abscissa: exposure time, varying somewhat from one humidity to another, but usually between the limits two to six hours. Ordinate: surviving cell fraction plotted logarithmically. Continuous curves: washed cells without additive. Dashed curves: with added sodium chloride. Rippled curves: with added lithium bromide.

effect of lithium bromide, on the other hand, appears to be rather less at 33% than it was at 75.3%. Most striking is the fact that at zero relative humidity both substances appear to stabilize the preparation. The experiments thus seem to suggest that the injurious effects of these substances are not brought about by their ordinary solutions, or if they are, only because of considerable abnormalities in behavior caused by the physical-chemical environment offered by the cell. Furthermore, even at zero humidity they both produce some effect, although in the opposite sense to that found at higher humidities. Whether this protection results from a physical barrier to residual water loss, or from the blocking of certain groups susceptible to lethal interaction, cannot be said at present.

Urea was chosen because it has saturated solution properties similar to sodium chloride, although having a radical and totally different type of biological action. Urea typically denatures proteins and inactivates biological systems at a rate that increases with a very high power of its concentration. Since in an ordinary aqueous solution the equilibrium concentration of urea is determined entirely by the humidity, and since the urea becomes dry at humidities below about 75%, it is easy on this simple basis to predict what ought to happen: if urea is added to a suspension of bacteria before they are placed on the membrane filter and dehydrated. As the humidity approaches the limiting value of about 75%, the humidity-dependent mortality rate should rise extremely steeply; then, as soon as the humidity passes the solubility cutoff point, urea should no longer have any effect at all upon the decay curves. Figures 15, 16, and 17 show what actually happened when such experiments were done. In the first place, after a constant time of two hours at a relative humidity corresponding to an equilibrium urea concentration of 18 molal, we see that the anticipated rapid decay does take place provided that we use enough urea; the decay rate seems to depend not solely on the concentration of urea at equilibrium but upon there being enough of the substance present. This is not altogether surprising, because all of these predictions would break down if the actual number of molecules of active substance available to each cell were insufficient to produce the lethal environment. Figure 16 shows much the same sort of thing, at a higher humidity corresponding to an equilibrium concentration of five molal. Here, if the initial concentration of urea in suspension is too low, there seems to be a delay in the decay process, or an induction period, whereas if the equilibrium concentration, five molal, is present in the suspension from the start, the induction period is either abolished or becomes much less pronounced, and for the first 2 or 3 hours an approximately first-order decay takes place. This sort of experiment can of course provide some evidence concerning the rate at which the equilibration with water vapor takes place in these membrane filter experiments, and what appears in Figure 15 as an induction period could be the result of a water vapor equilibration rate that is very much slower than we had supposed. On the other hand, we quite frequently observe an induction period also when concentrated urea solutions act upon Serratia marcescens in aqueous suspension, so that the effect may be due in some way to delayed penetration

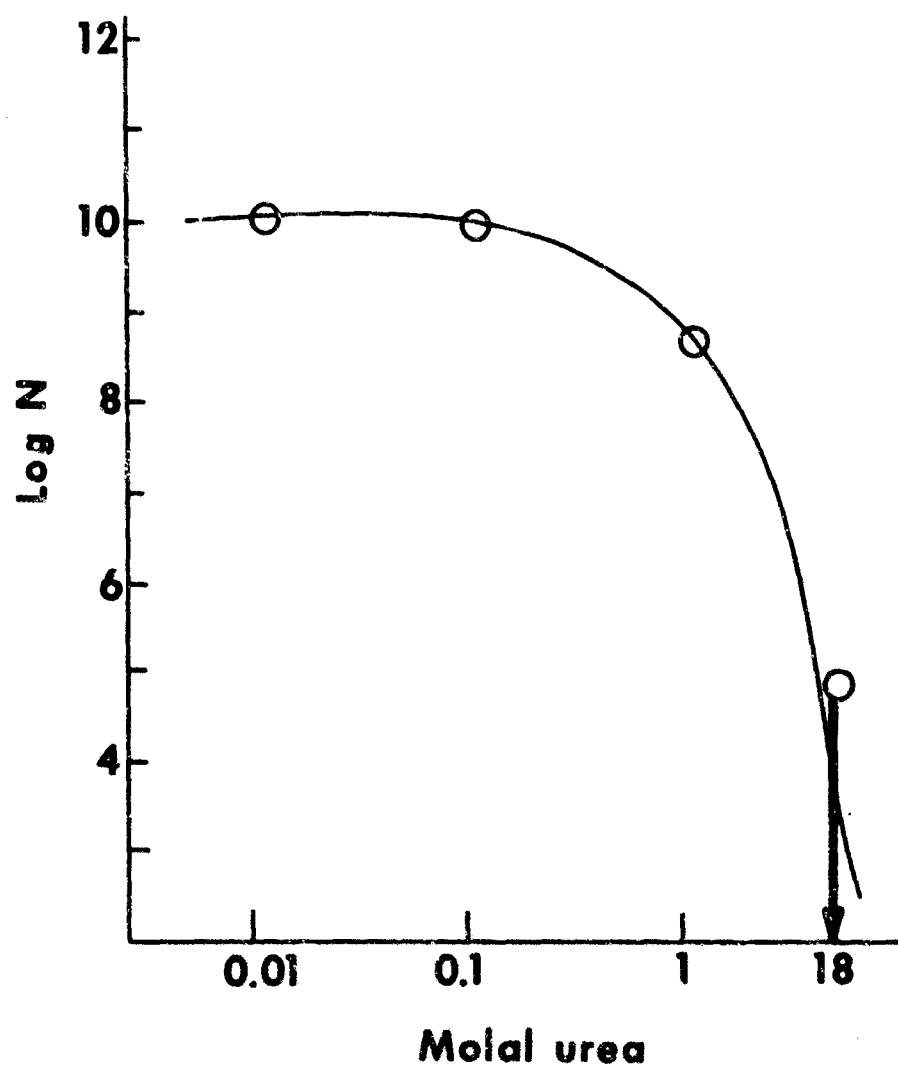


Figure 15. Effect of Urea on Survival of Washed *S. marcescens* Deposited on Membrane Filters and Exposed to a Relative Humidity of 76.4% for Two Hours. Abscissa: initial concentration of urea in test suspension before deposition upon membrane filters in logarithmic scale of molality. Ordinate: logarithm of surviving cell concentration. The equilibrium concentration of urea dissolved in water and at a relative humidity of 76.4% is roughly 18 molal.

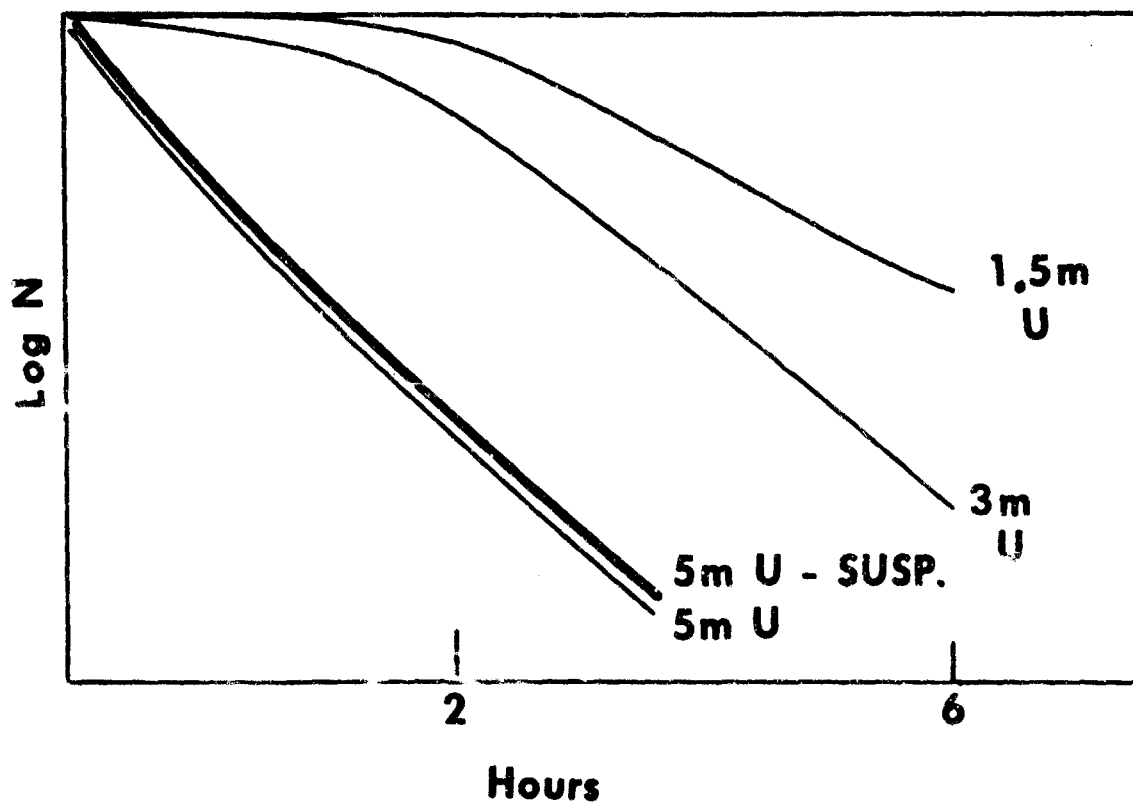


Fig. 16. Effects of 5 Molal Urea upon Washed *S. marcescens* in Membrane Filter Preparations and in Aqueous Suspension. Abscissa: exposure time in hours. Ordinate: logarithm of surviving cell fraction. In the membrane filter experiments suspensions of cells containing initially 1.5, 3, and 5 molal urea were deposited upon membrane filters and exposed to a relative humidity of 92.5%. The equilibrium urea concentration at this relative humidity is 5 molal. The experiment on the cell suspension was done by adding 5 molal urea and sampling the cells for enumeration at intervals.

of the urea into the cell or to preliminary changes that it must produce before any lethal effect can be seen. The figure shows one other interesting point that provides a good deal of support for the validity of our argument and also for the membrane filter technique. The decay curve for a preparation of Serratia marcescens in five molal urea equilibrated on a membrane filter at a relative humidity of 92.5% agrees very closely with the decay curves obtained when a suspension of similar cells is simply incubated in a water bath and sampled from time to time. Since these experiments were made, Mercer* has done a rather detailed study of the kinetics of killing of Serratia marcescens by urea as a function of temperature and concentration in ordinary aqueous suspension, and some of these findings may have to be qualified in the light of his more extensive and accurate data. Figure 17 summarizes the results obtained at different relative humidities. Here we see in much more striking form the same sort of failure in our prediction of a sharp humidity cutoff that we found with sodium chloride. At a relative humidity of 57.7%, well below the solubility limit for urea, the decay rate is scarcely distinguishable from that produced in the vicinity of the cutoff point and is actually greater than that found at a humidity of 86.3%. Nevertheless, although the cutoff does not occur sharply at the predicted point, it does occur gradually at much lower relative humidities, and when one gets down to 33%, the urea produces a significant but rather small increase in the rate of decay brought about by humidity change itself. At zero per cent the same type of phenomenon is seen that we found with sodium chloride and lithium bromide, namely, an apparent protection by urea. Since this has been seen with three somewhat sharply contrasting substances, including one of very high biological activity, it must have a rather nonspecific mechanism, possibly one of physical protection.

C. LONG-TERM DECAY CURVES FOR WASHED S. MARCESCENS

When the theoretical equations already discussed were first developed, we were encouraged to find that several sets of our data for the decay of S. marcescens at different relative humidities, and also in the presence of urea, could be fitted very satisfactorily to selected theoretical curves. We realized, however, that nothing conclusive could be said until better data, covering a much more extended range of exposure times, could be obtained. Although the membrane filter technique appeared to be well suited for experiments of this nature, a considerable number of experiments at several relative humidities have failed to give the sort of reproducibility that is necessary for adequate comparison with theoretical curves. As illustrated in Figure 18, duplicate measurements within the same experiment, and using of course the same parent cell

* W. B. Mercer. Personal communication.

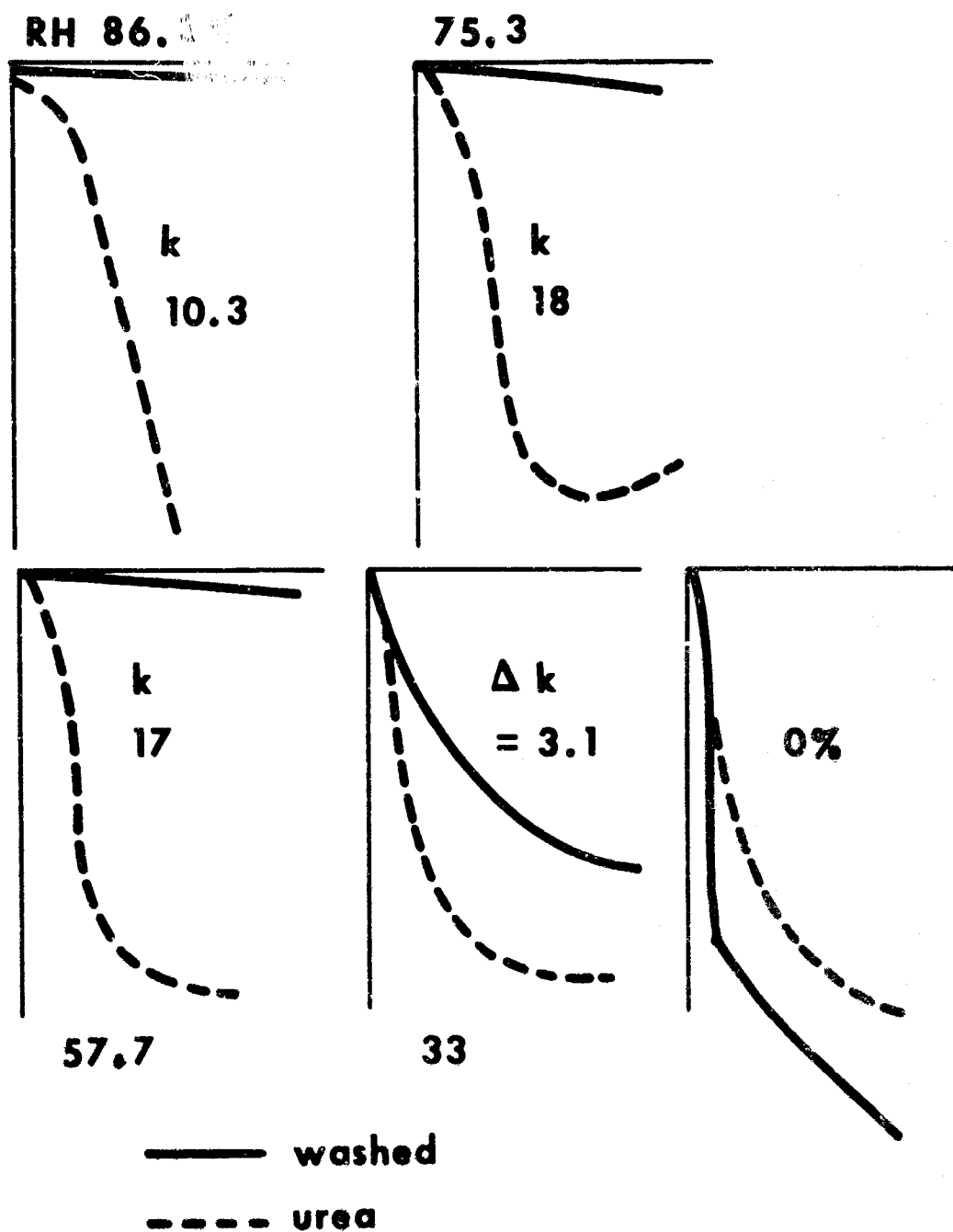


Figure 17. Effects of Urea on Membrane Filter Preparation of Washed *S. marcescens* at Various Relative Humidities. Abscissae: exposure time up to 2 or 3 hours. Ordinate: logarithm of surviving cell fractions. Continuous curve: washed cells without additive. Dashed curves: cells with added urea. The respective relative humidities and approximate first-order decay constants are indicated on the diagram.

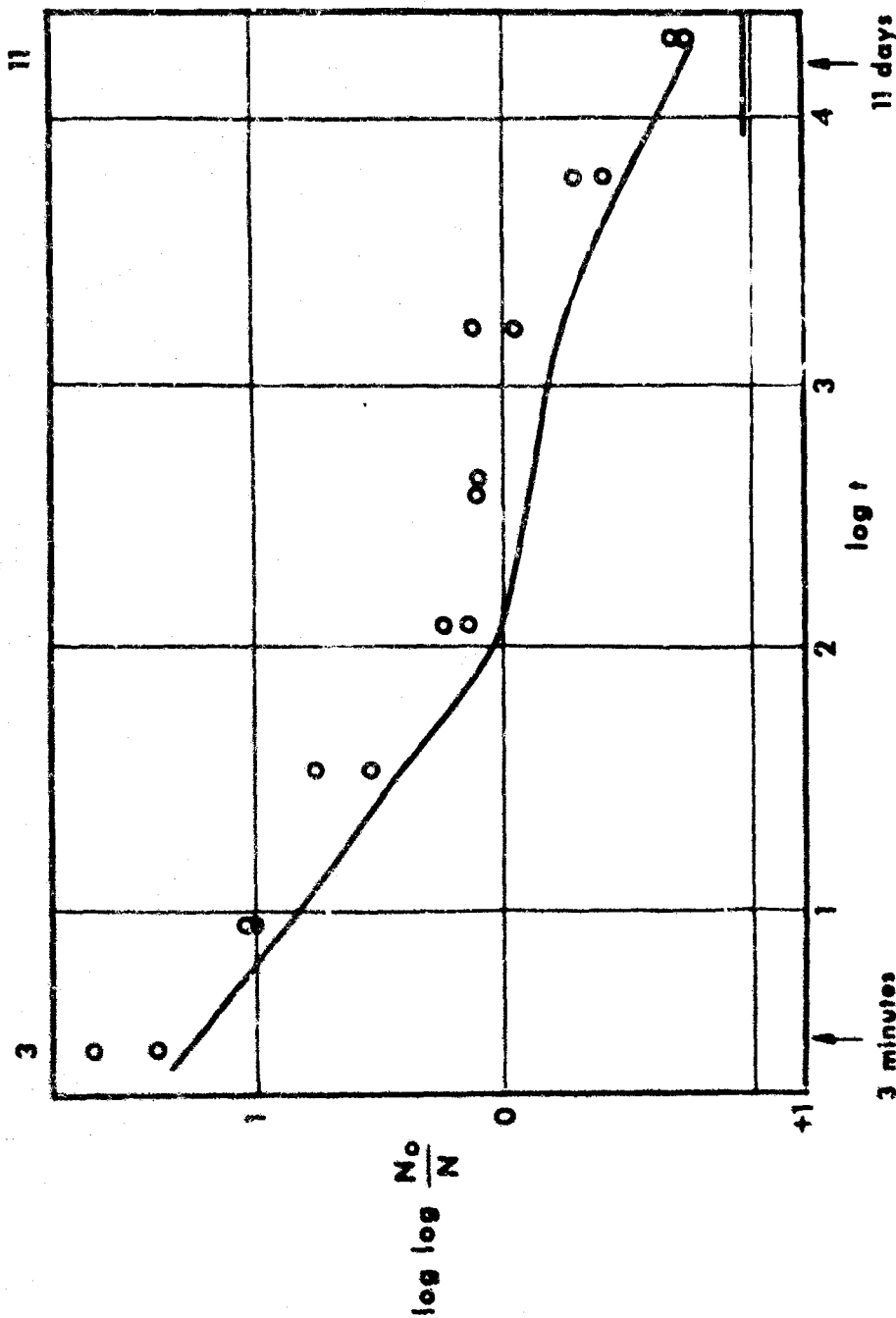


Figure 18. Long-Term Decay Curves for Washed *S. aureus* Exposed on Membrane Filters to a Relative Humidity of 33% at 25 C. Abscissa: logarithm of exposure time in minutes; a value 4.2 corresponds to an exposure of about 11 days, and one of about 0.5 to an exposure of 3 minutes. Double logarithm of decay ratio. Circles: results of duplicate exposures within a single experiment. Curve: averages of five experiments. Heavy line at lower right corner indicates one surviving cell.

suspension, are often in very satisfactory agreement; the real trouble comes when one tries to repeat the same curves either with the same cell suspension at a later date or with fresh cells prepared in exactly the same manner. Even when duplicate results within an experiment are in accord, there evidently is some uncontrolled factor leading to irregularity in the decay curve, so that the curve may appear rippled, although the ripples do not of course occur in the same places when the experiment is repeated. This only shows that one cannot hope under present circumstances to get reliable curves without combining the results of several experiments, and it is imperative therefore to improve the reproducibility from one experiment to the next. Some representative results are nevertheless included in Figures 19 and 20, showing respectively the effect of changing the growth medium, the change that occurred when an orange variant of the organism was obtained accidentally, and the contrasting effects of different humidities.

D. CONSEQUENCES OF EXCLUDING AIR AND OF PREVENTING CONTINUED WATER LOSS

We have been diverted temporarily from the study of long-term decay processes by the need to define our idea of "equilibration" with a water vapor atmosphere in more exact terms. We have assumed up to now, and with several good reasons, that gross equilibration occurs quite rapidly in a membrane filter experiment, since most of the intercellular water is blotted away quite quickly, and the amounts of water that have to be transferred thereafter from a rather small (10^8) collection of cells is not very great. In experiments on the adsorption isotherms of bacteria for water, Charles Stevens* found some years ago that equilibrium was only achieved after many hours and it is therefore quite likely that in any ordinary humidity experiment the kinetic picture is being complicated by what one might call residual water exchanges that would be very hard to detect by ordinary methods. If this is indeed the case, then a strong argument could be made in favor of carrying out measurements of decay kinetics at constant water content rather than at constant ambient humidity. In such an experiment, one would expose the cells to some appropriate humidity for enough time to accomplish withdrawal of the bulk of the water, and then one would in some way prevent further water loss and follow the decay process from that point on. In first trying to test this possibility we thought that further loss of water could very easily be prevented simply by covering the membrane filter and the bacteria with mercury. We found at once that the mercury had a very striking effect in protecting the cells from further mortality, and were at first inclined to think that this indicated slow residual water loss as one of the more important processes involved in the lethal event. Further experiments, however, showed that while the mercury prevented water loss

* Personal communication.

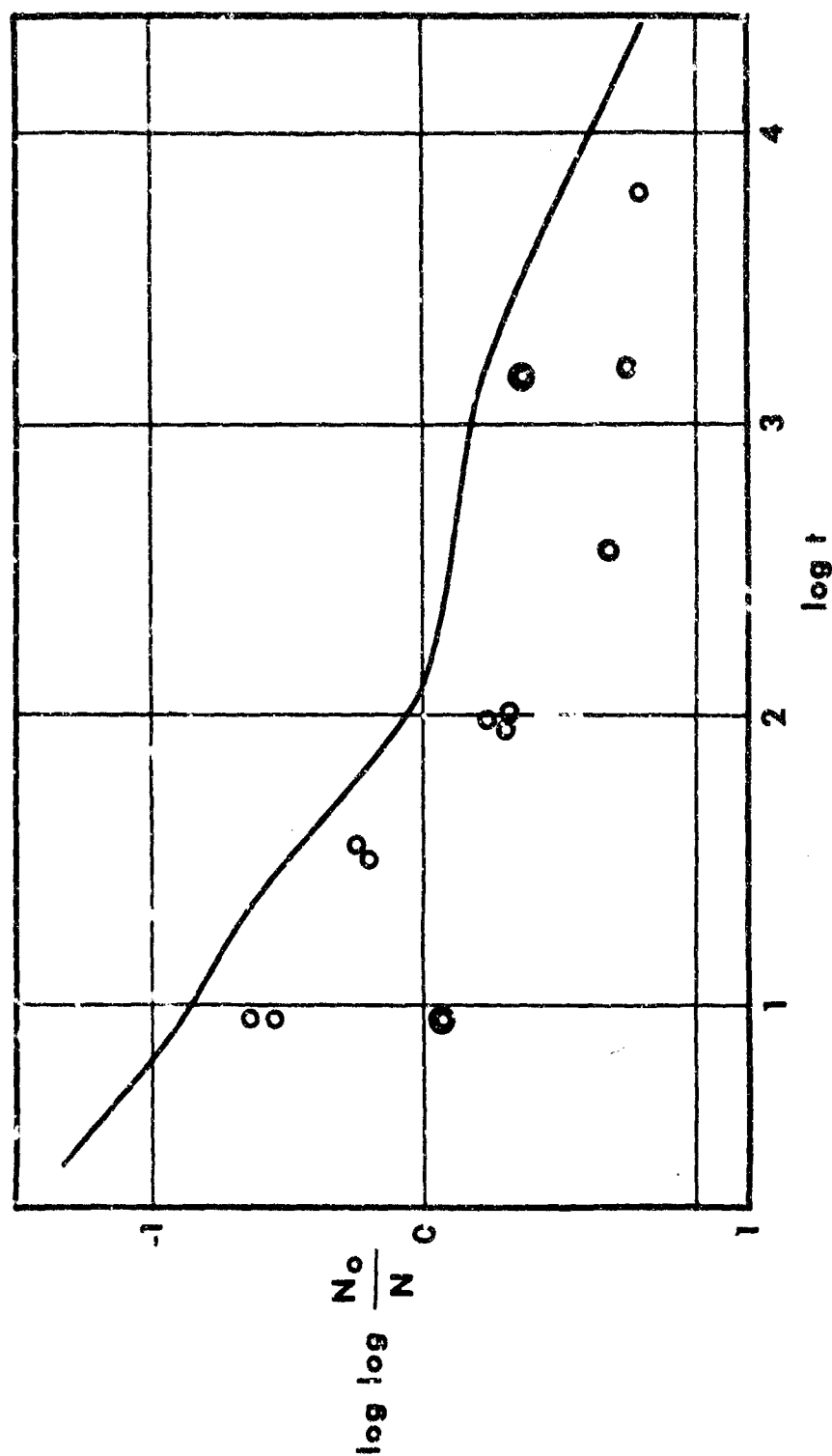


Figure 19. Long-Term Exposure of Washed *S. marcescens* Deposited on Membrane Filters to a Relative Humidity of 33%.
 Abscissa: logarithm of exposure time in minutes.
 Ordinate: double logarithm of decay ratio. Curve: average of five experiments as in Figure 18. Small light circles: data for an orange variety of the same strain of *S. marcescens*. Larger heavy circles: washed *S. marcescens* grown in Bunting's synthetic medium.

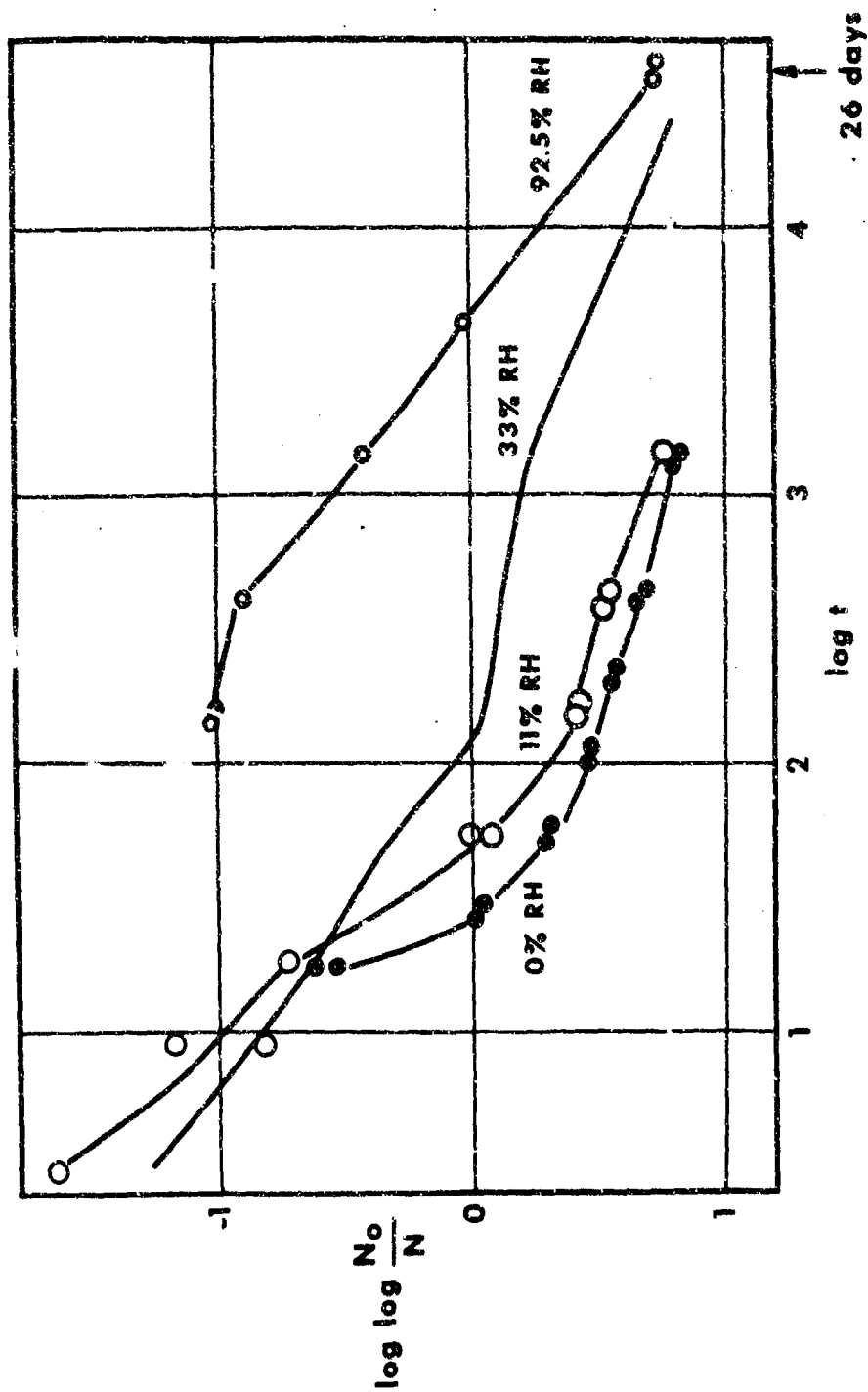


Figure 20. Prolonged Exposure of Washed *S. marcescens* Deposited on Membrane Filters to Various Relative Humidities. Abscissa: logarithm of exposure time in minutes; a value 4.6 on the abscissa corresponds to an experiment lasting about 26 days. Ordinate: double logarithm of decay ratio. Duplicate exposures within a single experiment are represented in each case except for the data at 33% relative humidity, which gives the average of five experiments as in Figure 18 and 19.

it also excluded air, and some, if not all of the protective effect noted was due to the latter. A number of experiments involving the use of exposure to different humidities in air and in vacuum, and involving the covering of cells with mercury both in air and in vacuum, have given us a rather complicated but reasonably consistent picture of what is happening. Some of the findings are summarized in Figures 21 and 22, which are purely schematic. In our earlier work some years ago we consistently found that at low humidities the cells died much more rapidly in vacuo than in air; this is shown, since it has been confirmed several times, in the two lower heavy lines of Figure 21. We now know that the reason for this finding lay in our attempts to do simple experiments by using a very sloppy kind of vacuum, namely that piped on in our laboratory. This provides a vacuum of a centimeter or two of mercury at best, and possibly permits diffusion of water vapor into the system. When the cells are subjected to a 30-micron vacuum, something resembling the top curve of Figure 21 is obtained; the decay rate is very greatly reduced by comparison with those resulting both from air and from the line vacuum. Evidently air is toxic, and evidently the line vacuum increases the decay rate in comparison with air by increasing the rate of removal of water while leaving a sufficiently high partial pressure of air to produce the toxic effect. If, now, the exposures to these three environments are suddenly cut off at some point by the addition of mercury, several things happen. (i) If the cells have been in a 30-micron vacuum and the vacuum is restored after adding the mercury, the results are rather variable, from a slight decrease in decay rate to a rather slight increase. If the air over the mercury is not removed by evacuation, then the mercury fails to protect the cells from decay and after some time the surviving fraction of cells is about the same as it would have been had the cells been exposed to air throughout the experiment. Again, if after the cells have been "protected" by mercury in vacuo for some time the mercury is removed and air is admitted, then there is once again a very steep loss of viability, as far as we can see, ending somewhere near the surviving fraction that would have been reached with continuous exposure to air. (ii) In the second case, where we add mercury after the cells have been exposed to air for a certain length of time, leaving the mercury at atmospheric pressure, there is a very substantial decrease in decay rate; but once again, if the mercury is removed after an hour or two and the cells are exposed to air for a few minutes, the viable count drops steeply to a point somewhere near the curve for air. The explanation for all this seems to lie in the toxicity of air, and certain properties of the reaction concerned can be deduced. When the cells are decaying in the presence of air, those parts of the system susceptible to attack by air are attacked as they are formed, or uncovered, or activated, or whatever the process may be, so that we have a moderately rapid, more or less uniform, loss of viability. On the other hand, if the cells are first dehydrated in a vacuum, where they are for the time being protected against the effects of air, they also build up a sort of "oxygen debt" or reservoir of oxidizable material, so that as soon as air is admitted in

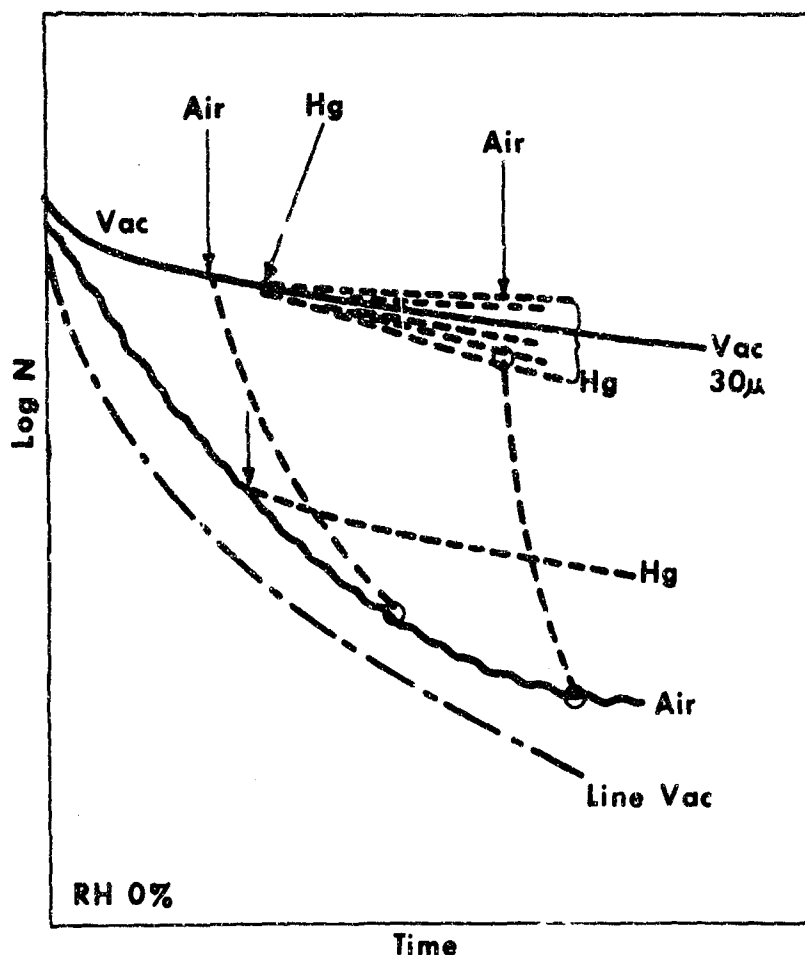


Figure 21. Schematic Representation of Effects when Washed S. marcescens, Deposited upon Membrane Filters, is Exposed at Different Elapsed Times to a Poor Vacuum, to a 30-Micron Vacuum, to Air, or Covered with Mercury. Relative humidity maintained at 0% throughout. Abscissa: elapsed time, up to 2 or 3 hours. Ordinate: logarithm of surviving cell concentration, spanning four or five logarithmic units. Continuous line labeled Vac 30 microns: decay curve for cells exposed to a 30-micron vacuum throughout. Rippled curve marked Air: decay curve when cells are exposed to air throughout. Broken line with dots labeled Line vac: decay when cells are exposed to a vacuum of several cm of mercury throughout. Vertical arrows indicate points at which air is admitted, in order to cover the cells with mercury or to study the effect of continued exposure to air. Diagrams show (i) the slight erratic effect of mercury when placed upon cells that have been exposed to a 30-micron vacuum only except for the time required to open the desiccator and apply the mercury; (ii) the accelerated decay that occurs when cells kept in a good vacuum are exposed to air; (iii) the protection afforded by covering with mercury when cells have previously been allowed to decay in air; and (iv) accelerated decay produced when cells are exposed throughout to a poor vacuum.

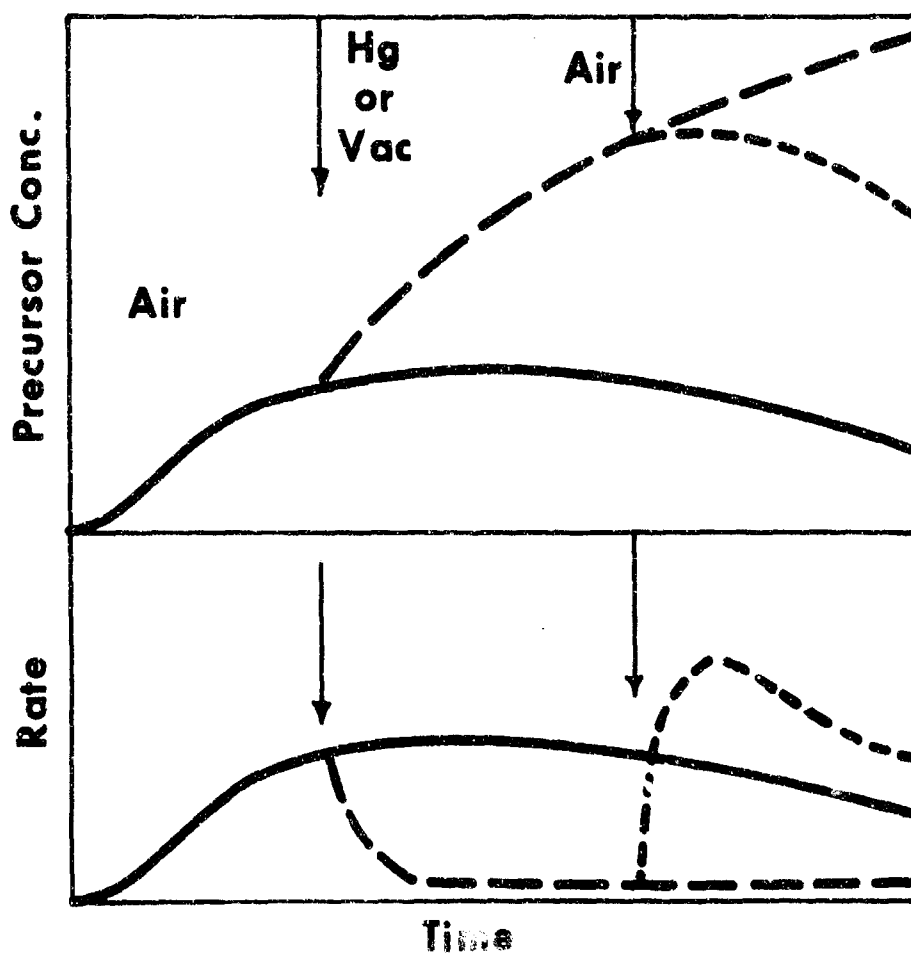


Figure 22. Schematic Illustration of Effects of Interrupting a Decay Process in Air by Covering Cells with Mercury or by Evacuating. The illustration is drawn in terms of the concentration of a hypothetical oxidizable precursor for a lethal substance produced by air. The upper part of the diagram, continuous curve, shows the limited accumulation of the precursor when the cells remain in air, a steady state being established between the rate of formation and the rate of oxidation of this precursor. When the cells are protected from air by mercury or a vacuum, this steady state is disturbed and further accumulation of precursor takes place, with the result that when air is readmitted the oxidation occurs at a considerably increased rate. The lower part of the diagram illustrates the presumed accompanying changes in rate of inactivation of the cells.

order to add the mercury they take up enough air to satisfy the "oxygen debt" and suffer the consequences of formation of the toxic product of oxidation. The same thing happens when the mercury is removed, after a nearly continuous period in vacuo, and the cells are exposed to air. A possible qualitative mechanism for these events in terms of concentration of an oxidizable substance and the resulting lethal steady state is given in Figure 23, which once again is explained in the legend.

While all this certainly suggests that we ought to regard the toxic effect of oxygen as one of the significant components in our inactivation scheme, it does not answer the question originally posed concerning the possibility of prolonged slow losses of water. Despite its interest and importance, the oxygen effect (or should I say the air effect, since we have not yet proved that oxygen is the villain in the case) is to us little more than a nuisance, to be avoided if possible. It would be quite different if the effect of air were of an all-or-none character, and if cells became completely and permanently stable in its absence; but this is not the case, and exclusion of air is evidently one quite effective way of reducing the number of operative lethal processes. We may also suspect that air has something to do with the very rapid initial losses that are encountered when the cells are placed upon the membrane filter, and by carrying out experiments in a controlled atmosphere throughout, substituting nitrogen, hydrogen, or other gases for air, we may very well be killing two birds with one stone. The question of slow losses of water can then be tackled as an important problem in its own right. It must be added that carrying out the entire procedure in the absence of air is not such an easy matter, and an alternative may be offered by the finding of Shon,* some time ago, that some degree of stability to aerosolization could be produced by, so to speak, exhaustively oxidizing the stock cell suspension by prolonged agitation with oxygen. On the other hand, the part possibly played by carbon dioxide in this procedure has not been elucidated to our knowledge, and we shall probably be better off if we can work in gaseous atmospheres controlled accurately with respect to such potentially important constituents as carbon dioxide.

* Milton Shon. Personal communication.

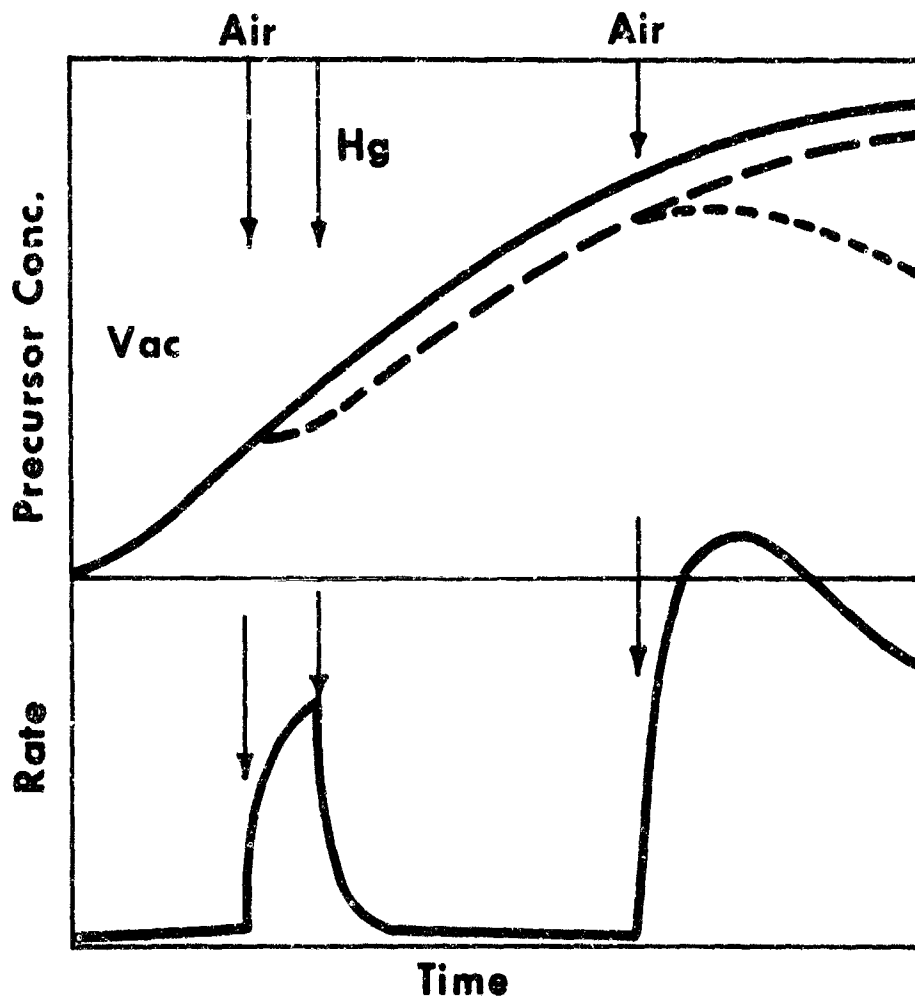


Figure 23. Accumulation of an Oxidizable Precursor of the Lethal Substance when Cells are Inactivated in a Vacuum and Later Exposed to Air. In the absence of other lethal processes, the rate of cell inactivation in a vacuum will be zero; whenever air is admitted, even briefly, rapid inactivation occurs through rapid oxidation of the accumulated precursor.

VI. PHYSICOCHEMICAL PROPERTIES OF CELLS RELEVANT TO THE DEHYDRATION PROBLEM

A. INTRODUCTION

It is conceivable that the reactions or interactions that lead to loss of viability are highly localized processes involving only a small number of critical molecules or groups within each cell. It is equally possible that statistically large numbers of loci are concerned, and in the absence of any method of pinpointing the exact biochemical reaction and its site of occurrence, there is a great need for information obtained by every possible method as to the state and nature, even if only a statistical average, of the cell's interior and its bounding structures. The possible approaches to this information are in varying degree somewhat indirect. We shall discuss briefly a few of those that seem to be of special interest in connection with the humidity problem, leaving others to deal with the more specialized types of measurement such as nuclear and electron spin resonance.

B. WATER UPTAKE ISOTHERM; HYSTERESIS; WATER-TRANSFER KINETICS

The water vapor isotherm of bacterial cells provides at least some indication of the average behavior of the cells, and possibly something more in the event that particular discontinuities can be established as a function of relative humidity. The phenomenon of hysteresis also provides a clue to what is going on as the cell takes up and loses water, and as more is known in detail of the order in which different types of groups are uncovered, the better are the chances for seeing some kind of correlation with mortality rates at different relative humidities. Of even greater potential interest is the question of the rate of water loss and water uptake, over the entire region from fully hydrated cells subjected to relatively small osmotic gradients down to extremely dry cells from which all but some minute and not very well defined fraction of water has been removed. At the end of the humidity scale, where the cells are nearly fully hydrated, of course, the approach has to be one involving permeability kinetics, and one in which the effects of permeating and nonpermeating solutes play a vital role. This kind of process, which usually occurs quite rapidly (sometimes in fractions of a second) is being investigated by Mr. Stanley Lovett, our guest from the Microbiological Research Establishment at Porton, England. The results are bound to be applicable to calculation of the time course of various events accompanying the dehydration of cells in this range, and therefore of the events that must occur initially in all types of dehydration. It may be of quite vital interest to know whether a particular solute outside the cell at the time of aerosolization has time to enter the cell before drying takes place, or whether it must remain outside; and by the same token it may be

important to know whether some intracellular substance able to pass through the membrane at a certain rate has a chance to leak out before the cell becomes dry. At lower humidities, in the regions where solutes are being precipitated out, and where adsorbed water dominates the picture, different approaches have to be used, but we feel that possibly a unified statement of phenomena covering the entire range of humidities may be possible and have indeed published a paper, with C.L. Stevens, E.L. Carstensen, and W.B. Mercer in which such a unified presentation is attempted. In this adsorption region, again, it is of interest to look at the kinetic picture, of course on a time scale much more extensive than that encountered in osmotic interchanges, and we know of little published work of this kind. It may be appropriate to report here a private statement by D.G. Dervichian of the Pasteur Institute, to the effect that he had observed discontinuities in the rate of water loss of proteins held at constant relative humidity. Here again, if the existence of such discontinuities could be confirmed for proteins and extended to microorganisms, an attempt to correlate them with patterns of loss of viability would be of great significance.

C. DIELECTRIC PROPERTIES OF DEHYDRATED CELLS

Measurement of the electrical conductivity and capacity of cell suspensions at low frequencies provides some information about the processes of conduction along, or through, the external structures such as the cell walls, and about the nature of the polarized elements existing in their vicinity. As the frequency is increased, to an increasing extent the alternating current is able, so to speak, to short-circuit the poorly conducting cell membrane, with the result that processes of conduction or capacitative charging within the cell become of increasing significance, so that something can be learned in a very direct manner about the state of the cell interior. Hermann Schwan and Edwin Carstensen and others have shown that dielectric measurements at high frequencies are quite feasible with dried proteins and amino acid powders, but extensive work on bacterial cells as a function of water content have yet to be carried out. The manner in which the magnitudes and the critical frequencies of the various dielectric dispersion regions might vary with water content would be a significant index to the state of the cell interior, and once again might prove to be capable of correlation with biological properties such as decay kinetics. At extremely high frequencies the dielectric decrement produced by cellular macromolecules might be turned into an increment if significant concentrations of amino acids or other small polar molecules were produced by cellular activity.

D. PHYSICAL CHEMISTRY OF SOLUTES IN PROTOPLASM

We have given some preliminary evidence that the humidity zones through which several substances are biologically effective do not agree with the known properties of their saturated solutions. In the case of urea it is

of course recognized that extensive interactions occur with many of the components of protoplasm, but this is not known in sufficient detail, especially as a function of urea concentration, to be of much value in providing an explanation for the behavior observed. In all these cases, and in many others, particularly for example in those involving specific effects of substances active at quite low concentrations, we would profit greatly by more detailed physicochemical knowledge of the behavior of these substances in the presence of biological materials. The method of choice in most cases would be the measurement of vapor pressure depression, and work of this kind has been initiated.

E. SPECTROSCOPIC AND LIGHT-SCATTERING METHODS

As a result of developments in our laboratory and on contract, there are indications that it may become feasible to obtain a reasonably reliable spectroscopic characterization of intact cells by methods of diffuse light spectrophotometry, and insofar as the various chromophores in bacterial cells are sensitive to the nature of their immediate environment, changes associated with dehydration and rehydration should be detectable. The scattering of light by these cell suspensions is also under investigation both by turbidimetric methods and by the study of the angular dependence of scattering; measurements of this kind may be made to impinge upon the dehydration problem in two ways. In the first place, removal and restoration of water may, by virtue of a kind of hysteresis, result in a redistribution of substances within the cell that may affect their light-scattering properties. Secondly, according to the observations of Lucille Smith, Lester P. Packer and others, the inception of certain metabolic activities by bacteria is sometimes accompanied by radical changes in the light-scattering properties, and these might be used to determine whether changes in the ability to initiate these metabolic processes might result from removal and restoration of water. This section of the discussion has been introduced mainly in the hope of showing the relevance of some of the kinds of physical investigation that are being done within our group, and to show that in conjunction with other biochemical studies these might ultimately contribute as much to the understanding of the effects of dehydration as the more direct attack that has been the principal subject of this paper.

VII. PROBLEMS

A number of problems have been touched upon in the foregoing discussion, and these need only be recapitulated here, in some sort of order of precedence:

1) Reproducibility of biological materials

The problem of first importance is that of getting reproducible test material. The number of possible variables is so great that an investigation of them would divert us from the main issue for a long time to come, and the great need is for some sort of inspired advice from the microbiologists, who might very well wish to suggest an entirely different organism and entirely different methods of preparing it.

2) Reproducibility of physical environment

Here the number of possibilities is perhaps less, but comparatively subtle factors such as carbon dioxide pressure may need more attention than has been given to them hitherto. In view of such recent discoveries as those of Lwoff and of Pollard, to the effect that in some systems, under some circumstances, changes of temperature of less than a degree may produce radical changes in the biological properties of the system, a closer control of temperature may be desirable.

3) Reduction of number of operative lethal interactions

Exclusion of air seems to be one method; there ought to be others, but whether by simplifying the conditions of growth of the cells, or by giving them a much richer diet, is by no means obvious.

4) Criteria of injury

The criteria of loss of viability must be evaluated, as well as the possible use of other criteria of biological injury.

5) Elimination of side effects

Side effects, such as initial losses associated with application of cells to membrane filters, must be eliminated.

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13. ABSTRACT Approaches to the study of lethal effects of dehydration and relevant experimental results obtained by the author and co-workers since 1958 are summarized informally. The paper includes (i) a working hypothesis of interacting lethal processes from which self-limiting long-term decay curves can be calculated. (ii) Preliminary experimental data illustrating the types of information needed for application of the hypothesis. (iii) An account of the preparation of "simulated aerosols" by deposition of cells upon membrane filters. (iv) Approaches to the identification of lethal interactions, including the correlation of lethal effects with the colligative properties of simple additives. (v) Illustrative data for <u>Serratia marcescens</u> in the presence of NaCl, LiBr, and urea. (vi) Data on short-term decay of washed <u>Serratia marcescens</u> at 13 ambient relative humidities. (vii) Preliminary data on the effects of oxygen in accelerating humidity-dependent decay and on possible effects of slow residual water transfer.		

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